The role of retinoic acid in the regulation of T helper welfare

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THE ROLE OF RETINOIC ACID IN THE REGULATION OF T HELPER CELL FATE

A thesis submitted to the School of Medicine at King's College London for the Degree of Doctor of Philosophy

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Declaration

The work presented in this thesis is my own and all experiments, except where acknowledged in the text, were performed by me.

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Abstract

In response to antigenic stimulation and extrinsic cytokine signals $CD4^+$ T cells differentiate into specialized effector T helper (Th) cell subsets. Regulation of plasticity between these $CD4^+$ T-cell lineages is critical for immune homeostasis and prevention of autoimmune diseases. However, the factors that regulate lineage stability are largely unknown. In Chapter 2, retinoic acid (RA), the active metabolite of vitamin A, is shown to regulate the stability of T helper 1 (Th1) cells, traditionally considered the most phenotypically stable $CD4^+$ T cell subset. Signaling through RA receptor α (RARα) sustains stable expression of Th1 lineage specifying genes, as well as repression of genes that instruct Th17 cell fate. RA signaling is shown to be critical for limiting Th1 cell conversion into Th17 effectors and for preventing pathogenic Th17 responses in vivo. These findings identify RA/RARα as a key component of the regulatory network governing maintenance and plasticity of Th1 cell fate and define a new pathway for the development of pathogenic Th17 cells. The molecular mechanisms underlying regulation of Th1 plasticity depend on the ability of RARα to recruit p300 to cis regulatory enhancers at key Th1 defining genes. Chapter 3 expands on these findings and examines a global role for RARα in the regulation of enhancers in both Th1 and Th17 cells. Strikingly, RARα is shown to be the dominant regulator of enhancer activation in Th1 cells. Comparison of RARα bound enhancers in T helper cell subsets reveal lineage specific RARα bound genes and provide a mechanistic basis for the cell context dependent effects of RA on T helper cell fate. Collectively, the data identify a fundamental role for RA synthesis and signaling in the epigenetic regulation of T helper cell

fate and suggest a broader role for RARα in the regulation of enhancers outside of the immune system.

Dedication

To Luke Streatfeild.

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To my parents for their endless support and inspiration.

List of Figures

Figure 21. (*) Identification of putative pioneer factors and RARα cofactors

List of Publications:

This thesis contains portions of text from the following published manuscripts:

1. Brown, C.C.;* Esterhazy, D.; Sarde, A.; London, M.; Pullabhatla, V.,;Osma-Garcia, I.; al-Bader, R.; Ortiz, C.; Elgueta, R.; Arno, M.; de Rinaldis, E.; Mucida, D.; Lord, G.; Noelle, R. (2015). Retinoic acid is essential for Th1 cell lineage stability and prevents transition to a Th17 cell program. *Immunity* 42:499-511 *co-corresponding author

2. **Brown, C.C.,** Noelle, R. (2015). Seeing through the dark: New insights into the immune regulatory functions of vitamin A. *Eur J Immunol* 45:1287–1295

Attributions:

Chapter 2

The experiments reported in Figure 7 of the published manuscript were performed and analysed by Dr. Daniel Mucida, Daria Esterhazy, and Mariya London. ChIP-sequencing was performed by Active Motif. Computational analysis of ChIP-seq data was performed by Venu Pullabhatla. Microarrays were performed by Matthew Arno and Miltenyi Biotec. Transcriptome analysis of dn*Rara* and WT Th1 cells was performed by Miltenyi Biotec.

Chapter 3

Computational analysis of ChIP-seq data was performed in collaboration with Venu Pullabhatla. Analyses were designed by me, save for Figure 14, and executed by Venu. GSEA, GO and KEGG analyses were performed in collaboration with Dov Pechenick. ChIP-sequencing was performed by Active Motif. Figures generated through collaborative work are marked by (*) in the List of Figures.

List of abbreviations

AP-1	Activating protein-1
APC	Antigen presenting cell
BATF	Basic leucine zipper transcription factor
BSA	Bovine serum albumin
ChIP	Chromatin immunoprecipitation
CNS1	Conserved non-coding sequence 1
DC	Dendritic cell
dnRARα	dominant negative retinoic acid receptor α
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
GALT	Gut associated lymphoid tissue
GSEA	Gene set enrichment analysis
H3K4me1	Monomethylation of histone 3 lysine 4
H3K4me3	Trimethylation of histone 3 lysine 4
H3K27me3	Trimethylation of histone 3 lysine 27
H3K27ac	Acetylation of histone 3 lysine 27
IEL	Intraepithelial lymphocyte
IFN	Interferon
IL	Interleukin
IRAK	IL-1 receptor-activated kinase
IRF	Interferon-regulatory factor
iTreg	Inducible regulatory T cell
Jak	Janus kinase
LDTF	Lineage defining transcription factor
LLO	Listeriolysin O
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
OVA	Ovalbumin
PCR	Polymerase chain reaction
PP	Peyer's Patch
qPCR	quantitative Polymerase chain reaction

pTreg	Peripheral regulatory T cell
RA	Retinoic acid
RARE	Retinoic acid response element
RARα	Retinoic acid receptor alpha
RXR	Retinoid X receptor
SE	Super-enhancer
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TF	Transcription factor
Tfh	Follicular helper T cell
TGF- β	Transforming growth factor-β
Th	T helper cell
Treg	T regulatory cell
iTreg	Inducible T regulatory cell
pTreg	Peripheral T regulatory cell
tTreg	Thymic regulatory T cell
VA	Vitamin A
VAD	Vitamin A deficient
WT	Wild-type
YFP	Yellow fluorescent protein

CHAPTER 1: Epigenetic and transcriptional regulation of T helper cell fate: a role for retinoic acid

The crosstalk between the innate and adaptive arms of the immune system creates an inflammatory microenvironment that ultimately controls the fate of responding T cells. Naïve $CD4^+$ T-cells circulate between blood and the lymphatic system, passing through secondary lymphoid organs where they encounter antigen presenting cells (APCs) bearing antigens. Encounters with cognate antigens within these environments drive differentiation of naïve $CD4^+$ T cells into functionally distinct effector T helper (Th) and regulatory T cell (Treg) subsets. The APC along with other innate immune cells present at the site of T cell priming provide extracellular signals through cytokines and other mediators. These activate complex networks of transcription factors (TFs) in a spatiotemporal manner to drive coordinated changes in gene expression and direct the differentiation program towards a specific T cell lineage. Appropriate lineage specification of naïve $CD4^+$ T cells is critical to a successful immune response. Lineage commitment and maintenance of cell fate involves the interplay of multiple transcription factors and epigenetic modifications which together direct cell-specific gene expression programs (Tsankov et al., 2015). Although traditionally thought of as distinct lineages, over the last few years there has been increasing appreciation of developmental flexibility between T helper cell subsets both in vitro and in vivo. Dysregulated $CD4^+$ T cell responses underlie the pathogenesis of allergic and autoimmune diseases, and thus underscore the importance of understanding the pathways that drive lineage specification and plasticity.

Transcriptional regulation of T helper cell specification

Cytokines direct T helper cell specification, and in turn T helper cell subsets produce distinct patterns of cytokines which allow T cells to recruit different cells with appropriate effector functions, tailored to the pathogen. Th1 cells produce Interferon-γ (IFN-γ) and are important for host resistance to intracellular pathogens. Th2 cells produce Interleukin-4 (IL-4), IL-13 and IL-15 and help to control parasitic infections through promotion of B cell class switching to IgE. Th17 cells produce IL-17A, IL-17F, IL-21 and IL-22 and play a role in mucosal immunity to bacteria and fungi through recruitment of neutrophils but have also been implicated in various autoimmune diseases. $CD4^+$ T cells can also differentiate into Treg cells with immunosuppressive function. These cells can either arise directly in the thymus (tTreg), or from naïve T cells in the periphery (pTreg). Treg cells differentiated in vitro are also referred to as inducible Treg (iTreg) cells. Treg cells are distinguished by the production of transforming growth factor-β (TGF-β) which suppresses unwanted immune responses to commensals, self-antigen and innocuous environmental antigens encountered at barrier sites. In addition to these well-characterized $CD4^+$ T-cell subsets, new lineages are emerging, defined by novel cytokine profiles and distinct transcriptional regulators, for example Th9 and the enigmatic follicular T helper (Tfh) cell subset (Hatzi et al., 2015; Veldhoen et al., 2008). These subsets are considered distinct lineages based on the selective expression of 'master regulator' transcription factors, which are necessary and sufficient for lineage specification. T-bet is selectively expressed in Th1 cells, Th17 cells express RORγt, Th2 cells express GATA3, and Treg cells are defined by Foxp3

expression (Fontenot et al., 2003; Ivanov et al., 2006; Szabo et al., 2000; Zheng and Flavell, 1997).

The concept of a 'master regulator' implies that a single transcription factor can impart the phenotype of its respective lineage. However, this oversimplifies the transcriptional landscape of T helper cells. Chromatin immunoprecipitation coupled with genome wide sequencing (ChIP-seq) has identified networks of transcription factors which dictate T cell phenotype (Ciofani et al., 2012). These transcription factors are deployed in a tightly controlled spatiotemporal manner to direct gene expression programs specific for $CD4^+$ T cell subsets in response to extracellular cues.

Environmental signals direct T helper cell fate

Extracellular cues are key determinants of naïve $CD4^+$ cell fate. Cytokines signal through the janus kinase–signal transducer and activator of transcription (Jak-STAT) signaling pathway. Naïve $CD4^+$ T cells constitutively express basal levels of STAT proteins. Signaling through cytokine receptors leads to phosphorylation of the relevant STAT family member in the cytosol resulting in dimerization and translocation to the nucleus to bind to target cis-regulatory elements and initiation of lineage specific gene expression (O'Shea et al., 2011). Thus, STATs operate as signal dependent transcription factors, converting extracellular events to induction of genes which drive transcription factors.

Central to the role of STATs in lineage specification is the induction of the relevant 'master regulator' transcription factor. In Th1 cells, early expression of T-bet following TCR activation is dependent on IFN-γ induced STAT1 activation, whereas late expression of T-bet, post termination of TCR signaling, has been shown to be dependent on IL-12/STAT4 (Schulz et al., 2009). Th2 differentiation is instructed by IL-4 activation of STAT6 which in turn drives expression of GATA3 (Kaplan et al., 1996). Induction of the Th17 pathway is more complex as the Th17 lineage shows considerably heterogeneity, in part due to the number of cytokine conditions involving combinations of IL-6, TGF-β1, IL-1 and IL-23 that can drive Th17 differentiation in vitro (Basu et al., 2013; Veldhoen et al., 2006). IL-6 appears to be critical for Th17 specification and is the principal activator of STAT3 in the early phase of Th17 differentiation. Th17 cells produce IL-21 which serves to maintain STAT3 signaling, providing autocrine regulation of the Th17 phenotype. IL-1 promotes the NF-κB transcription factor IRAK (IL-1 receptor-activated kinase) which enhances phosphorylation of STAT3 (Mills et al., 2013). More recently it was shown that IL-1 can amplify STAT3 signaling through inhibition of SOCS3, a negative regulator of STAT3 signaling (Basu et al., 2015). In the same study, IL-1 was shown to be critical for Th17 responses in vivo, resolving a long-standing controversy over the requirement for IL-1 in Th17 differentiation. Th17 development is closely intertwined with iTreg differentiation; these two cell-types share a requirement for TGF-β (Bettelli et al., 2006). In the absence of IL-6, TGF-β together with IL-2 drives the conversion of naïve T-cells to $Foxp3^+$ Tregs (Chen et al., 2003; Mucida et al., 2007; Xiao et al., 2008). IL-2 activates STAT5 which binds to the Foxp3 promoter and this pathway is critical for iTreg

differentiation (Davidson et al., 2007) IL-2/STAT5 signaling also enhances Th1 and Th2 differentiation (Liao et al., 2011).

Lineage stability vs. plasticity

Despite being classified as lineages, the presence of hybrid $CD4^+$ T cells which co-express cytokines and transcription factors from different subsets suggests that a considerable degree of plasticity exists between $CD4^+$ T cell lineages (O'Shea, 2010). Early during differentiation, Th17 cells exhibit transient flexibility with co-expression of Foxp3 and RORγt, and at later stages of development Th17 cells can be readily converted to “Th1 like” cells expressing T-bet and IFN-γ, as well as Tfh and regulatory $IL\text{-}10^+$ (Tr1) cells (Gagliani et al., 2015; Garefalaki et al., 2011; Hirota et al., 2013; Lee et al., 2009). Conversely, transdifferentiation of Treg cells to Th17 cells has also been demonstrated in response to inflammation (Komatsu et al., 2014). Recent studies have identifed Treg populations that co-express lineage-specific transcription factors from effector T cell subsets, including T-bet, GATA3 or Bcl6 (Chaudhry et al., 2009; Chung et al., 2011; Koch et al., 2009; Zheng et al., 2009). These hybrid phenotypes provide functional advantages, enabling Tregs to control subset specific responses. For example T-bet expressing Treg cells express the chemokine receptor CXCR3 and can home to sites of Th1 mediated inflammation (Koch et al., 2009). Th1 and Th2 cells were traditionally considered more stable relative to their Th17 and Treg counterparts. This was challenged by a recent study demonstrating reprogramming of in vitro generated Th2 cells toward a Th1 phenotype in response to viral infection in

vivo (Hegazy et al., 2010). To date, no studies have identified plasticity of Th1 cells.

Although developmental flexibility between $CD4^+$ T cell subsets is functionally advantageous, providing breadth to the immune response and sensitivity to changes in the extracellular environment, dysregulated $CD4^+$ T cell responses underlie a number of immune mediated diseases including allergic and autoimmune disease. IFN-γ^+ Th17 cells have been implicated in several human autoimmune diseases including inflammatory bowel disease (Annunziato et al., 2007), juvenile idiopathic arthritis (Nistala et al., 2010), and multiple sclerosis (Kebir et al., 2009); ex-$Foxp3^+$ Th17 cells play a pathogenic role in rheumatoid arthritis (Komatsu et al., 2014); and IL-17^+ Th2 cells have been positively linked to the severity of asthma (Irvin et al., 2014). Elucidation of the developmental pathways for these hybrid cells and identification of the factors that regulate $CD4^+$ T cell plasticity will allow the development of therapeutic strategies to restore imbalances between T helper cell subsets observed in disease states.

Counter regulatory transcriptional networks maintain T-cell commitment

Maintenance of T-cell lineage commitment requires suppression of factors which instruct alternative cell fates. Lineage defining 'master regulators' and STATs not only enforce a particular lineage but also serve to antagonize alternative cell fates. For instance, STAT5 and STAT3 reciprocally regulate iTreg and Th17 differentiation (Basu et al., 2015; Laurence et al., 2007). Their reciprocal action is achieved in part through competitive antagonism of DNA

binding. T-bet directly binds to GATA3 and these transcription factors share a set of binding targets, suggesting competitive regulation of gene expression (Kanhere et al., 2012). T-bet also inhibits Th17 differentiation by competitively inhibiting Runx1 binding and activation of *Rorc* (Lazarevic et al., 2011). RORγt and Foxp3 exhibit extensive overlap in their DNA targets suggesting reciprocal regulation of Th17 and iTreg programs (Xiao et al.,2014). Thus, maintenance of a particular T helper cell phenotype requires both stable expression of lineage defining transcription factors and active repression of responsiveness to cytokines that can instruct opposing factors.

Epigenetic regulation of T helper cell lineage specification

Instruction of divergent cell fates by lineage defining transcription factors requires the induction of distinct transcriptional networks resulting in temporal changes in gene expression. In order for transcription factors to mediate changes in gene expression, they must first bind to their cognate DNA binding motif at *cis*-regulatory elements in the genome. Epigenetic alterations in the chromatin landscape are critical in determining accessibility of DNA to transcription factor binding. A number of chromatin modifications have been identified that delineate 'active' accessible and 'repressive' inaccessible chromatin. Recent technological advances in the study of genome wide epigenetic modifications have highlighted the cell specific nature of epigenetic modifications that accompany transitions in cell fate (Hawkins et al., 2013; Vahedi et al., 2015; Wei et al., 2102). Thus, cellular differentiation requires coordinated changes in transcription factor expression and recruitment of epigenetic regulators to chromatin.

Chromatin modifications can broadly be divided into two categories: covalent modifications to histone proteins such as acetylation, phosphorylation and methylation; and DNA methylation (Calo and Wysocka, 2013; Ziller et al., 2013). These chromatin modifications are mediated by an array of coactivator and corepressor proteins possessing enzymatic activity. These proteins do not possess DNA binding activity and their site of action must be determined by transcription factors that are able to bind *cis* elements in the DNA and at the same time recruit epigenetic modifiers through protein-protein interactions.

Selection of cell type specific enhancers

Distal *cis*-regulatory regions termed enhancers are genetic elements that have the potential to enhance transcription levels from gene promoters, often located hundreds to kilo-base pairs from the enhancer element. Enhancers function as binding platforms for clusters of lineage-determining transcription factors and signal-dependent transcription factors which drive enhancer activation, inducing looping of the enhancer to its target promoter and delivery of enhancer associated factors (Calo and Wysocka, 2013). Genome wide mapping of enhancer elements has revealed cell-type specific patterns of active enhancer elements with dynamic changes in the complement of enhancers during cell differentiation (Lara-Astiaso et al., 2014; Hawkins et al., 2013). Binding of transcription factors in response to cell signaling events occurs primarily at enhancers that are already poised for activation (Heintzman et al., 2009; 2007), leading to the idea that cell type specific gene expression is determined by the enhancer landscape.

In order to establish an active enhancer a number of steps must occur. A widely accepted model is that “pioneer” factors with the ability to bind nucleosomal DNA bind to primed enhancer elements marked by H3K4me1, and facilitate binding of other transcription factors by nucleosomal depletion and recruitment of chromatin modifiers to generate accessible, ‘permissive’ enhancers (Rothenberg et al., 2013). Binding of the relevant transcription factors to accessible regions leads to recruitment of coactivators such as the histone-aetyltransferases (HATs) p300 and CBP (Chan and La Thangue, 2001). This is followed by recruitment of RNA polymerase II (Pol II) and transcription from the enhancer region to generate enhancer RNAs (De Santa et al., 2010; Kim et al., 2010). eRNAs are critical for enhancer functionality and allow looping of the enhancer to the promoter through an as yet unidentified mechanism. Acetylation of H3K27 (H3K27ac) by HATs not only increases the accessibility of enhancer regions, but plays a role in transcription as H3K27ac was recently shown to regulate the activity of RNA Pol II (Stasevich et al., 2014).

Advances in next-generation high-throughput DNA sequencing have enabled mapping of the epigenetic landscape. Using a combination of histone modifications (H3K4me1 and H3K27ac) as well as p300 binding it is possible to identify enhancer elements on a genome wide scale (Heintzman et al., 2009; Rada-Iglesias et al., 2010). Elements marked by monomethylation of histone H3 lysine 4 (H3K4me1) in the absence of histone acetylation are considered to indicate ‘permissive’ enhancers. The presence of p300 together with H3K27ac indicates an active enhancer (Rada-Iglesias et al., 2010) where the presence of

H3K27ac correlates with enhancer activation and levels of eRNA transcripts (Creyghton et al., 2010). In embryonic stem cells (ESCs), p300 may be bound in the absence of H3K27ac but together with the repressive mark, H3K27me3 (Rada-Iglesias et al., 2010). These enhancers are considered to be 'poised' for activation.

Super-enhancers regulate cell identity

The distribution of cell-specific enhancers is asymmetric with some genes located in regions with multiple enhancers. Although there are thousands of putative enhancers in any given cell type, there are only a few hundred regions marked by clusters of enhancers, spanning several thousand base pairs, so called 'super-enhancer' (SE) regions (Whyte et al., 2013). Whilst typical enhancers govern the majority of genes expressed by a particular cell type, genes with high levels of cell type specific expression are enriched for super-enhancers suggesting that these regions are important for cell identity. The importance of super-enhancers is demonstrated by the finding that cell-specific super-enhancers are enriched for SNPs associated with diseases relevant to that cell type (Vahedi et al., 2015). A recent study of super-enhancers in $CD4^+$ T cell subsets identified enrichment of super-enhancers at cytokine loci and transcriptional regulators (Vahedi et al., 2015). These findings suggest that disruption of transcription factor binding at enhancers can have a profound impact on T cell responses.

Enhancer activation in T helper cells

Given the importance of enhancers in cell specific gene expression, the mechanisms that underlie lineage specific enhancer activation during T cell differentiation have been an area of intense investigation. TCR signaling induced transcription factors, such as NFAT and activating protein-1 (AP-1), are thought to act as pioneer factors although it is not clear how these common signals select lineage specific enhancers. The best-characterised pioneer factors are BATF and IRF4 which co-operatively bind cis-regulatory elements in non-polarised, activated $CD4^+$ T cells and facilitate binding of the core Th17 transcription factors: RORγt and STAT3 (Ciofani et al., 2012). The expression of BATF and IRF4 is upregulated following TCR stimulation, independent of the cytokine milieu, suggesting a potential pioneering role for these transcription factors in other T helper cell lineages. However $BATF^{-/-}$ mice have no overt impairment in Th1 responses, arguing against this model (Schraml et al., 2009). Temporal studies of transcription factor binding are required to dissect out the hierarchy of transcription factor binding at primed enhancers in the remaining T helper cell subsets.

The pioneer factors may be elusive, but recent studies have shed light on downstream events required for enhancer activation. Lineage-specific enhancers are enriched for the relevant lineage-determining transcription motifs, pointing towards a potential role for these factors in enhancer activation. Surprisingly, the master regulators, T-bet, GATA3 and RORγt, were found to have only a minor role in regulating the enhancer landscape (Vahedi et al., 2012; Xiao et al. 2014). Instead, studies in Th1 and Th2 cells suggest that

STAT proteins play a global role in enhancer activation, as cells deficient in the relevant STAT protein exhibited loss of p300 at enhancers (Vahedi et al., 2012). In Th2 cells, H3K4me1 deposition was also affected by loss of STAT6 and in a separate study, STAT6 occupancy at enhancers was found to precede enhancer activation during Th2 differentiation (Hawkins et al., 2013). STAT6 may therefore be a pioneer factor in Th2 cells. It is unclear to what extent STAT proteins can directly recruit p300 or whether they co-operate with other factors with the ability to modify chromatin.

An epigenetic basis for T helper cell plasticity

Epigenetic modifications not only permit transcription factor binding and downstream transcriptional activation but can also inhibit transcription factor activity. Repressive marks associated with closed chromatin structure prevent access of TFs to their target genes. Thus, epigenetic modifications can reinforce cell fate decisions and raise barriers that must be overcome for cells to adopt alternative phenotypes in response to extracellular cues. This was highlighted by genome-wide mapping of active and repressive marks H3K4me3 and H3K27me3 respectively, in $CD4^+$ T cell subsets (Wei et al., 2102). Gene loci for the relevant lineage-specific TFs and cytokines are marked by H3K4me3 while repressive marks are present at genes associated with alternative T-helper cell subsets. Bivalent domains marked by both H3K4me3 and H3K27me3 are considered to be 'poised' for activation or repression. An epigenetic basis for plasticity observed in Th17 cells and iTregs is suggested by the presence of bivalent markers at transcription factor and cytokine loci associated with alternative cell fates (Wei et al., 2102). One caveat to the

interpretation of these studies is the use of bulk cell populations for ChIP-seq. It is possible that bivalent domains represent two populations of cells with either H3K4me3 or H3K27me3 at a particular locus rather than the co-existence of these marks within a single cell. Single-cell epigenomic profiling is required to address this question.

A further example of an epigenetic basis for $CD4^+$ T cell plasticity is observed in Treg cells. iTreg and tTreg both express Foxp3. However, iTregs exhibit phenotypic instability, readily adopting gene expression profiles associated with effector T cell subsets. Examination of the epigenetic landscape at the *Foxp3* locus reveals distinct chromatin modifications. The *Foxp3* locus remains methylated in iTregs, similar to naïve $CD4^+$ T-cells, whereas tTregs can be distinguished by a demethylated *Foxp3* locus (Huehn et al., 2009). These studies have enhanced our understanding of T helper cell differentiation and have spurned a wealth of interest in the epigenetic mechanisms that regulate T helper cell fate.

Enhancers maintain lineage stability

Epigenetic modifications are preserved through rounds of cell division and may explain the stability of gene expression changes induced by signal-dependent transcription factors, long after the initiating extrinsic cues are removed from the environment. Temporal examination of enhancer states during human Th1 and Th2 differentiation showed that enhancers remain active during late phase of differentiation, suggesting that enhancers are required for stable gene expression and maintenance of cell fate commitment (Hawkins et al., 2013). At

present, it is not clear whether enhancers are required to initiate transcription and/or sustain gene expression. Given that cellular plasticity requires the remodeling of enhancer regions in response to extracellular cues, it has been proposed that epigenetic regulators play a critical role in regulating lineage plasticity or stability.

Nuclear hormone signal dependent transcription factors

What is clear from the study of enhancer activation in cells outside of the immune system is that multiple signals are required for enhancers to become fully functional. As well as recruitment of p300, a number of coactivator complexes are required for enhancer functionality. No single transcription factor can drive enhancer activation, rather a complex of transcription factors collaboratively bind in a temporal order through DNA and protein-protein interactions. The ability of STAT proteins to shape the enhancer landscape points to a potential role for other signal dependent transcription factors in translating changes in the microenvironment to chromatin. Potential candidates include nuclear hormone receptors which are activated by a diverse group of ligands including hormones, vitamins and lipids. Of particular interest are the retinoic acid receptors. This is because retinoic acid (RA), the active metabolite of vitamin A, is actively synthesized at sites of T cell priming and has been shown to regulate cellular differentiation in a number of tissues (Guo et al., 2015).

Vitamin A: an essential nutrient for immunological health

Vitamin A, through its active derivative retinoic acid (RA), plays a critical role in embryogenesis, determining cell lineage and fate commitment (Niederreither and Dollé, 2008). In areas where malnutrition is endemic, vitamin A deficient (VAD) children have an increased burden of infectious disease (Sommer et al., 1986), highlighting the importance of vitamin A for immunity. The pivotal discovery that RA was constitutively synthesized by gut dendritic cells (DCs) was closely followed by several studies showing that RA was able to enhance induction of iTreg cells. These findings led to the view that RA might act to promote oral tolerance and shifted the attention away from the critical nature of RA in peripheral, effector immune responses. In recent years a broader role for RA in systemic immunity has re-emerged. Several studies have demonstrated regional induction of RA synthesis and signaling upon inflammation (Guo et al., 2012; Pino-Lagos et al., 2011), and RA has been shown to play an essential role in Th1 responses in allograft rejection, vaccination and gut infection (Hall et al., 2011; Pino-Lagos et al., 2011). These recent advances have shed light on a broader role for RA in directing T-helper cell fate, outside of the mucosal immune system.

Retinoic acid is synthesized at sites of inflammation

Multiple isoforms of RA exist. Of these, all-*trans* retinoic acid (ATRA) is the predominant biological form. RA is generated from retinol, which circulates in the plasma bound to retinol binding protein. RA synthesis is restricted to cells that express the enzymes required for conversion of retinol to RA. First, retinol

is converted to retinal by retinol dehydrogenase (RDH). Studies in *rdh*10$^{-/-}$ mice suggest that RDH10 is the critical isoform for retinal synthesis (Napoli, 2012). Retinal is then irreversibly converted to RA by one of three retinaldehyde dehydrogenase isoforms: RALDH1, RALDH2 or RALDH3 encoded by *aldh1a1*, *aldh1a2*, and *aldh1a3*, respectively (Lin et al., 2003; Niederreither et al., 1999). A role for RA in mucosal immunity was established by the discovery that dendritic cells within the mesenteric lymph node (MLN) and Peyer's patches (PP) constitutively express *aldh1a2* and *aldh1a1* respectively, and that RA could imprint gut tropism on T-cells through the induction of gut-homing receptors, CCR9 and $\alpha4\beta7$ (Iwata et al., 2004). Within both human and murine MLNs, DCs that express the highest levels of *aldh1a2* are the CD103^{+} subset (Coombes et al., 2007; Jaensson et al., 2008). Recently, it was shown that expression of 4-1BB, a member of the TNF receptor superfamily, correlates with CD103 positivity in dendritic cells and 4-1BB is therefore also able to identify MLN DCs with the highest levels of *aldh1a2* (Lee et al., 2012). Triggering of 4-1BB induced RALDH activity in vitro and 4-1BB deficient MLN DCs have weak RALDH activity pointing to a functional role for 4-1BB in the induction of RA synthesis.

Outside of the gut, examination of peripheral DC subsets have identified DCs expressing *aldh1a2* residing in the lung and skin, pointing to a role for RA in steady state immune responses at barrier sites (Guilliams et al., 2010; Gyöngyösi et al., 2013). Although the majority of peripheral DCs express negligible or low levels of RALDH, the identification of cytokines and pathogen-associated molecular patterns that can induce RALDH expression indicates that

RA synthesis and signaling may be a widespread occurrence during the course of a peripheral immune response. Treatment of splenic DCs with zymosan, a TLR-2 agonist, results in the induction of *aldh1a2* in vitro, and stimulation of WT but not TLR2$^{-/-}$ splenic DCs with *Candida albicans* has a similar effect (Manicassamy and Pulendran, 2009). In vitro, GM-CSF can induce both bone marrow-derived DCs and splenic DCs to express *aldh1a2* (Yokota et al., 2009).

Several *in vivo* studies have now demonstrated local induction of RALDH activity amongst DCs in response to a diverse array of inflammatory stimuli including viral infection, alloantigen and tumour burden (Guo et al., 2012; Wang et al., 2014),(Allie et al., 2013; Mielke et al., 2013; Pino-Lagos et al., 2011). In addition to RA synthesis by DCs, upregulation of *aldh1a2* expression has been observed in alternatively activated macrophages following infection with the helminth *Shistosoma mansoni* (Broadhurst et al., 2012). These studies suggest that RA synthesis and signaling may be a universal feature of immune responses both in the gut and the periphery. Intriguingly, peripheral induction of T and B cell responses in the presence of RA still leads to induction of gut homing receptors (Hammerschmidt et al., 2011; Wang et al., 2014). Induction of CCR9 expression on lung derived CD4^{+} T-cells following intranasal influenza infection resulted in trafficking of these cells to the small intestine. Local production of IFN-γ resulted in alterations to the gut microbiota which in turn led to increased numbers of intestinal Th17 cells. These findings explain the incidence of intestinal side effects observed in influenza patients but the functional relevance of gut homing to the primary immune response remains to be determined. Recent discoveries have shown that systemic immune

responses at sites distal to the gut are modulated through the gut microbiota (Lee et al., 2011; Wu et al., 2010) and it is possible that lymphocyte trafficking through the gut is a necessary rite of passage for effector T-cells. Regional RA production at peripheral sites of inflammation with subsequent induction of gut homing properties on lymphocytes may play a key role in shaping the course of the immune response. Regardless of the significance of RA in directing gut homing, the overall message from these studies is that RA synthesis and signaling at sites outside of the mucosal tissue, points to a global role for RA in the regulation of $CD4^+$ T-cell mediated immune responses.

RA regulation of T helper cell fate and plasticity

RA enhances iTreg differentiation and Treg stability

Following the initial study that identified RA synthesis by gut DCs, several groups went on to show that RA could dramatically enhance the TCR-TGF-β-mediated conversion of naïve $CD4^+$ T cells to iTreg in vitro (Benson et al., 2007; Coombes et al., 2007; Mucida et al., 2007). TGF-β mediated Foxp3 induction is dependent on Smad3 (Tone et al., 2008; Zheng et al., 2010). In addition to directly regulating the expression of Smad3 (Xiao et al., 2008), RA regulation of Foxp3 expression is in part mediated by binding of RAR/RXR heterodimers to a RA response element (RARE) in the enhancer 1 (CNS1) region of the Foxp3 gene, which facilitates binding of phosphorylated Smad3 to the enhancer region (Xu et al., 2010). The first in vivo evidence supporting a role for the RA/TGF-β-Smad3 pathway in the generation of pTreg cells in the GALT comes from a recent study utilizing mice lacking the Smad3 binding site within the CNS1

region. Aged mice develop deficiencies in Foxp3$^+$ cells in the LP and PP; however, the functional significance of this is unclear since no negative impact of the pTreg deficiency was observed in T-independent or T-cell-dependent models of colitis (Schlenner et al., 2012). Further studies are required to understand the in vivo contribution of RA to immune tolerance, both in the gut as well as at peripheral sites of immune responses.

In addition to enhancing TCR-TGF-β mediated Foxp3 expression, RA has also been shown to confer increased stability of Foxp3 expression in the face of inflammatory cytokines and co-stimulation amongst both iTreg and tTreg cells (Benson et al., 2007; Xiao et al., 2008) (Zhou et al., 2010). iTregs generated in the presence of RA express reduced levels of the receptor for IL-6, a Th17 instructing cytokine (Hill et al., 2008). Recently, RA and TGF-β were shown to induce expression of the microRNA miR-10a, which in turn inhibited expression of the Tfh master transcription factor, Bcl-6 (Takahashi et al., 2012). Conversion of Treg to Tfh cells has been described in PP (Tsuji et al., 2009), and miR10-a overexpression in iTreg cells was able to reduce this conversion in vivo (Takahashi et al., 2012). RA may therefore reinforce lineage stability by regulating opposing pathways that instruct alternative T helper cell fates.

A newly described role for RA in the conversion of CD4$^+$ T-cells to CD4$^+$CD8αα$^+$ T cells within the intestinal epithelium provides a further mechanism by which RA promotes anti-inflammatory responses within the gut (Reis et al., 2013). *Cd4^{cre}Rosa26$^{dnRara/dnRara}$* mice conditionally expressing a dominant negative form of the retinoic acid receptor RARα in CD4+ T-cells

(henceforth referred to as dn*Rara* mice) have severely impaired numbers of $CD4^+CD8\alpha^+$ IELs, which appear to play a critical role in preventing intestinal inflammation. dn*Rara* $CD4^+$ T cells fail to upregulate T-bet, which is required for induction of Runx3. Runx3 inhibits ThPok allowing reprogramming of $CD4^+$ cells towards $CD8\alpha^+$ IELs. The ability of RA to regulate expression of T-bet, the key Th1 transcription factor, hints at a broader role for RA in the regulation of T helper cell responses.

Retinoic acid is critical for $CD4^+$ effector Th1 responses in vivo

A role for RA in the generation of Treg cells seemed counterintuitive given the wealth of epidemiological data supporting a role for RA in immunity to infectious disease. Emerging data, which examines the effects of RA on effector lymphocytes in vivo, have gone someway to resolving this paradox by demonstrating a broader role for RA in T helper cell responses. Our own studies using dn*Rara* mice demonstrate a critical role for RA in the development of Th1 mediated immunity in a model of allograft rejection (Hall et al., 2011; Pino-Lagos et al., 2011). In keeping with the ability of RA to regulate Th1 differentiation, VAD mice infected with *Toxoplasma gondii* were found to have significantly reduced Th1 cells in their GALT and spleen (Hall et al., 2011). In the same study, VAD mice were also shown to have deficient Th1 cell responses in response to vaccination with ovalbumin (OVA) and *E. coli* toxin.

The molecular mechanism through which RA controls Th1 cell fate remains unclear. In vitro experiments conducted with $CD4^+$ T cells isolated from $RAR\alpha^{-/-}$ mice suggest that disruption of RA signaling leads to impaired

activation induced proliferation (Hall et al., 2011). However, dn*Rara* expressing $CD4^+$ T cells have normal proliferative capacity (Pino-Lagos et al., 2011). One possible explanation for this discrepancy is that unliganded RARs inhibit transcription through recruitment of corepressors (Kurokawa et al., 1995). In this case, deletion of RARα could lead to a loss of inhibition of RAR targets, and a paradoxical up-regulation of RA target genes in the absence of RA. In zebrafish, deletion of RAR isoforms resulted in a paradoxical increase in RA signaling with compensatory increases in expression of alternative RARs (D'Aniello et al., 2013). RARα does appear to be the critical receptor in mediating the effects of RA on $CD4^+$ T-cells as deletion of RARγ in haematopoietic cells had no impact on the development of Th1 responses following infection with *Listeria monocytogenes* (Dzhagalov and Chambon, 2007). **To date, no studies have examined transcriptional targets of RARα on a genome wide scale in Th1 cells and the precise mechanisms underlying RA/RARα regulation of Th1 differentiation remain undefined. Work described in Chapter 2 will address this question.**

In contrast to a physiological role for RA in supporting Th1 differentiation, RA treatment of naive $CD4^+$ T-cells polarised under Th1 conditions *in vitro* inhibits IFN-γ production (Iwata et al., 2003). It is unclear whether this reflects suppression of the full Th1 program through inhibition of T-bet or a limited effect of RA on cytokine production and/or secretion. RA has been shown to inhibit cytokine production by memory T cells in a non-lineage-specific manner. RA may therefore play distinct temporal roles during T cell differentiation, enhancing or limiting effector function.

Retinoic acid shapes Th17 responses

In addition to shaping Th1 responses, RA has also been shown to regulate Th17 differentiation. The earliest studies linking RA to TGF-β mediated Foxp3$^+$ expression identified a reciprocal role for RA in the inhibition of Th17 cells differentiated from naïve CD4$^+$ T cells with TGF-β and IL-6 in vitro. This appears to be in part due to reduced expression of IL-6R and IL-23R in responder T-cells (Xiao et al., 2008). Interestingly, the inhibitory effects of RA on Th17 differentiation are most prominent in the presence of IL-2 (Takahashi et al., 2012). IL-2 activation of STAT5 antagonises STAT3 and RA inhibition of Th17 differentiation is dependent on STAT5 (Takahashi et al., 2012; Xiao et al., 2008). Consistent with these in vitro findings, RA administration in vivo inhibits the development of Th17 responses and disease severity in experimental autoimmune encephalitis (EAE), a Th17 mediated autoimmune disease (Xiao et al., 2008). While pharmacological doses of RA inhibit Th17 generation, physiological levels of RA (<10nm), have been shown to enhance Th17 generation in vitro (Takahashi et al., 2012). In support of an in vivo role for RA in the generation or maintenance of Th17 cells, studies in VAD mice describe a near absence of Th17 cells both during homeostasis and in response to immune-mediated inflammation (Hall et al., 2011; Wang et al., 2010). However, two recent studies have identified a role for RA in the generation of type 3 innate lymphoid cells (ILC3s), which mirror Th17 cells in their cytokine profile. VAD mice and mice treated with an RA antagonist had impaired ILC3 responses both in steady state and in response to intestinal infection with *Citrobacter rodentium* (Spencer et al., 2014). ILC3s produce Th17 associated

cytokines and their absence in VAD mice may contribute to the impaired development of Th17 cells at the mucosal surface. Further studies are required in dn*Rara* mice to establish the cell intrinsic role of RA/RARα in Th17 generation. **Studies in Chapter 3 will start to address a role for RARα in Th17 differentiation.**

The absence of ILC3s in VAD mice has widespread implications for adaptive immune responses since a subset of ILC3s - lymphoid tissue inducer (LTi) cells - play a critical role in the development of secondary lymphoid organs and Peyer's patches. Maternal vitamin A and fetal RA signaling were found to directly regulate the expression of RORγt, instructing the development of LTis (van de Pavert et al., 2014). Mice exposed to an RA-deficient environment in utero had smaller secondary lymphoid organs which persisted in adult life resulting in impaired adaptive immune responses. Since many earlier studies have used VAD as a model for studying RA regulated dynamic T-cell responses, these studies must be re-evaluated in light of the widespread homeostatic defects observed in these mice.

Role of retinoic acid in Th2 differentiation

In addition to Th1 and Th17 mediated immune responses, RA synthesis has been reported in parasite infections suggesting a possible role for RA in shaping Th2 responses. Dietary VA levels have been shown to affect Th2 responses. For instance, enhanced production of Th2-associated cytokines, and concomitant reduction in IFN-γ, are observed in $CD4^+$ T cells isolated from *Trichinella spiralis* infected VAD mice (Cantorna et al., 1994). However, a

further twist is provided by the earlier study examining ILC dysregulation. In this, VAD induced expansion of ILC2s, leading to enhanced ILC2 derived Th2 type cytokines (Spencer et al., 2014). The presence of ILC2s appears to be critical for Th2 allergen responses in vivo (Halim et al., 2014). Thus, previously reported effects of dietary RA on Th2-cell responses in VAD mice may not be due to direct effects of RA on T cells. Studies in which RA levels are manipulated through administration of RA are conflicting with both enhanced and reduced Th2 responses reported in murine models of asthma following RA treatment (Schuster et al., 2008; Wu et al., 2013).

The preponderance of data from in vitro experiments, in which dose titration comparisons were performed on Th1, Th2 and Th17 polarisation, suggests dose dependent effects of RA on haematopoietic cell fate (Iwata et al., 2003; Takahashi et al., 2012; Uematsu et al., 2008). The seemingly paradoxical, opposite effects of RA on T cell fates may therefore be simply explained as concentration-dependence. This would allow T cells to act as an environmental sensor, with RA concentration being the signal. Further studies of RA gradients within lymphoid tissue are required to test this hypothesis. However, the overriding message is that administration of RA either in vitro or to vitamin A replete hosts in vivo may not provide insight into the physiological actions of RA on T-cell responses. Genetic approaches allow better appreciation of the impact of RA signaling on defined lineages of hematopoietic cells and will help to clarify the physiological role of RA in the regulation of T helper cell fate.

Temporal regulation of cell fate by retinoic acid

A further layer of complexity surrounds RA regulation of T helper cell fate with bi-phasic effects of RA on Th1 and Th2 differentiation reported in vitro (Iwata et al., 2003). In Th1 differentiation, induction of T-bet is dependent on IFN-γ/STAT1 signaling whereas maintenance of T-bet expression is dependent on IL12/STAT4 activation (Schulz et al., 2009), suggesting distinct roles for RA in the regulation of these signaling pathways. Similarly, a bi-phasic model for Th17 differentiation is emerging with early commitment dependent on TGF-β and IL-6 signaling, but maintenance and stability dependent on IL-21 and IL-23 signaling (Basu et al., 2013). Temporal regulation of cell fate by RA also hints at a possible role for RA in regulation of epigenetic modifications which are activated in a spatio-temporal manner during cell differentiation.

Retinoic acid signaling: novel mechanisms of transcriptional regulation

All-trans RA, the biologically active form of VA, signals through heterodimers of the RA receptors (RARs) and retinoid X receptors (RXRs) (Chambon, 1996). These receptors belong to the nuclear hormone receptor family. There are three isotypes for both RAR and RXR: α, β and γ. RAR/RXR heterodimers bind to RA response elements RAREs in target genes and act as ligand dependent transcription factors. RAR/RXRs mediate transcriptional regulation through the binding of corepressor or coactivator complexes dependent on the presence of ligand. The widely accepted view is that unliganded RAR/RXR heterodimers inhibit transcription of their target gene, through recruitment of co-repressors such as nuclear receptor corepressor (NCoR) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT). Binding of ligand to RAR leads to

release of corepressors and recruitment of coactivators such as p300 and CBP to the AF-2 domain with subsequent transcriptional activation of the target gene (Nagy and Schwabe, 2004). In addition, RA can mediate gene repression through recruitment of ligand-dependent corepressors, RIP140 and PRAME, which recruit histone deacetylases to RAR/RXR complexes to repress their activities (Epping et al., 2005; Perissi et al., 2010). RARs can also modulate transcription indirectly, through inhibition of transcription factor complexes, such as AP-1, although the underlying mechanisms remains uncertain (Nicholson et al., 1990).

In addition to its classical role as a transcriptional regulator, recent studies in ESCs have identified RA-RARα as an epigenetic regulator. Retinoids have been shown to regulate epigenetic changes including histone modifications and DNA methylation through the recruitment of coactivators with chromatin modifying properties (Gudas, 2013). p300 and CBP possess histone acetyltransferase activity and mediate acetylation of H3K27, a marker of active cis-regulatory regions. The emerging data implicating RARα in lineage plasticity suggest a potential role for RA/RARα in the regulation of the epigenome. **Work described in Chapters 2 and 3 will investigate this possibility.**

Given that RA is synthesised at sites of T-cell priming, RARα can act as a signal dependent transcription factor, responding to changes in the extracellular environment that becomes licensed during T cell activation. The ability of RARα to regulate epigenetic modifcations suggests RARα as a candidate molecule for regulation of enhancer landscape in T helper cells.

Intriguingly, TCR-signaling is required to observe RA mediated effects on lymphocytes pointing to a potential co-operative relationships between RARα and TCR-induced transcription factors, such as NF-kB or AP-1 family members. Alternatively, TCR-induced pioneer factors may be required for RARα to bind to its relevant target genes.

Summary

Studies of transcriptional changes and chromatin modifications paint a picture of tissue-specific programs orchestrated with astonishing precision. Despite intense investigation, the precise mechanisms by which enhancers transition to full competency during T cell differentiation remain unclear. Selective activation of new enhancer sites during cell-fate transitions is driven by lineage-specific factors, yet it remains unclear how TFs bound at enhancers regulate epigenetic changes to achieve fine-tuned alterations in gene expression. Chapter 2 examines the molecular basis for RA/RARα mediated regulation of Th1 differentiation and presents data identifying a role for RARα as a regulator of enhancers at key Th1 genes. Chapter 3 then explores a broader role for RARα as an epigenetic regulator and examines cell specific actions of RARα.

CHAPTER 2: PUBLICATION

Retinoic Acid Is Essential for Th1 Cell Lineage Stability and Prevents Transition to a Th17 Cell Program

Article

Immunity

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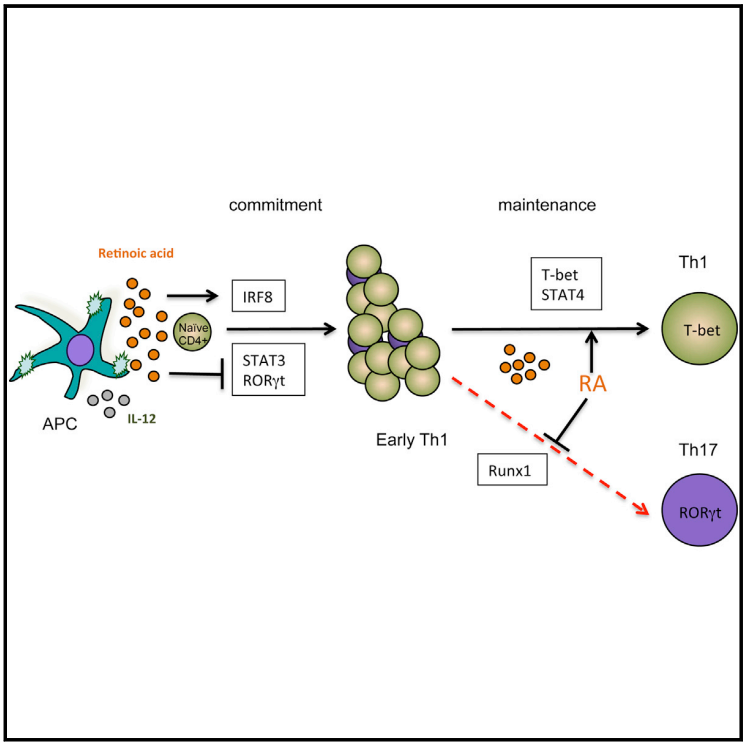
Graphical Abstract



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In Brief

Maintenance of T helper (Th)-cell identity is critical for appropriate immune responses; however, the factors that regulate Th-cell plasticity are unresolved. Brown et al. show that retinoic-acid signaling confers Th1 cell stability and restrains their conversion to Th17 cells.

Highlights

- Retinoic acid (RA) stabilizes Th1 fate commitment
- Signaling through RA receptor α (RARα) activates enhancers of Th1-cell-lineage-specifying genes
- RA-RARα represses Th17-cell genes in Th1 cells and constrains Th1-cell plasticity
- RA-RARα prevents development of pathogenic Th17 cells in vivo

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Article

Retinoic Acid Is Essential for Th1 Cell Lineage Stability and Prevents Transition to a Th17 Cell Program

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SUMMARY

CD4$^+$ T cells differentiate into phenotypically distinct T helper cells upon antigenic stimulation. Regulation of plasticity between these CD4$^+$ T-cell lineages is critical for immune homeostasis and prevention of autoimmune disease. However, the factors that regulate lineage stability are largely unknown. Here we investigate a role for retinoic acid (RA) in the regulation of lineage stability using T helper 1 (Th1) cells, traditionally considered the most phenotypically stable Th subset. We found that RA, through its receptor RARα, sustains stable expression of Th1 lineage specifying genes, as well as repressing genes that instruct Th17-cell fate. RA signaling is essential for limiting Th1-cell conversion into Th17 effectors and for preventing pathogenic Th17 responses in vivo. Our study identifies RA-RARα as a key component of the regulatory network governing maintenance and plasticity of Th1-cell fate and defines an additional pathway for the development of Th17 cells.

INTRODUCTION

Functional plasticity within cells of the innate and adaptive immune system increases the breadth of response to pathogens while also limiting responses detrimental to the host. CD4$^+$ T cells diversify into distinct effector subsets upon antigenic stimulation. Cytokines and other microenvironmental factors present during T-cell priming direct differentiation via induction of lineage specifying transcription factors (TFs): T-bet is the "master" regulator for T helper 1 (Th1) cells, RORγt for Th17 cells, and GATA3 directs the Th2 program. In vivo, the presence of cells that express TFs and cytokines from opposing Th lineages indicates flexibility between those subsets. Late-stage developmental plasticity is potentially perilous: interferon-γ (IFN-γ$^+$) Th17 cells have been implicated in several human autoimmune diseases including inflammatory bowel disease (Annunziato et al., 2007), juvenile idiopathic arthritis (Nistala et al., 2010), and multiple sclerosis (Kebir et al., 2009); ex-Foxp3$^+$ Th17 cells play a pathogenic role in rheumatoid arthritis (Komatsu et al., 2014); and interleukin-17 (IL-17$^+$) Th2 cells have been positively linked to the severity of asthma (Irvin et al., 2014). Elucidating the developmental pathways for these hybrid cells and identifying the factors that regulate Th-cell stability are therefore of critical importance.

Initial lineage specification is driven by cytokines, which activate signal transducer and activator and transcription (STAT) proteins: expression of T-bet is driven by IFN-γ-STAT1 and IL-12-STAT4 (Schulz et al., 2009); RORγt by STAT3 downstream of IL-6, IL-21, and IL-23 (Zhou et al., 2007). Less is known about the molecular mechanisms that sustain lineage identity. Epigenetic modifications stabilize gene expression and as such, are thought to play a key role in the maintenance of cell-fate commitment. However, the factors that co-ordinate chromatin changes with evolving TF networks in differentiating Th cells are not fully defined. One candidate is the vitamin A metabolite, retinoic acid (RA). RA is known to play a key role in directing the lineage fate of hematopoietic stem cells (Chanda et al., 2013), dendritic cells (DCs) (Klebanoff et al., 2013), innate lymphoid cells (ILCs) (Spencer et al., 2014), and CD4$^+$ T cells (Reis et al., 2013) through activation of the nuclear RA receptor (RAR). In addition to its classical role as a transcriptional regulator, recent studies in embryonic stem cells have identified RA-RAR as an epigenetic regulator (Kashyap et al., 2013; Urvalek and Gudas, 2014). RA synthesis is dynamically controlled at sites of T-cell priming during inflammation, where RA signaling on T cells has been demonstrated (Aoyama et al., 2013; Pino-Lagos et al., 2011). These studies suggest a potential role for RA in Th-cell plasticity. Indeed, RA is critical for Th1-cell immunity (Hall et al., 2011; Pino-Lagos et al., 2011) and RA has also been implicated in Th17-cell differentiation where its impact appears to be

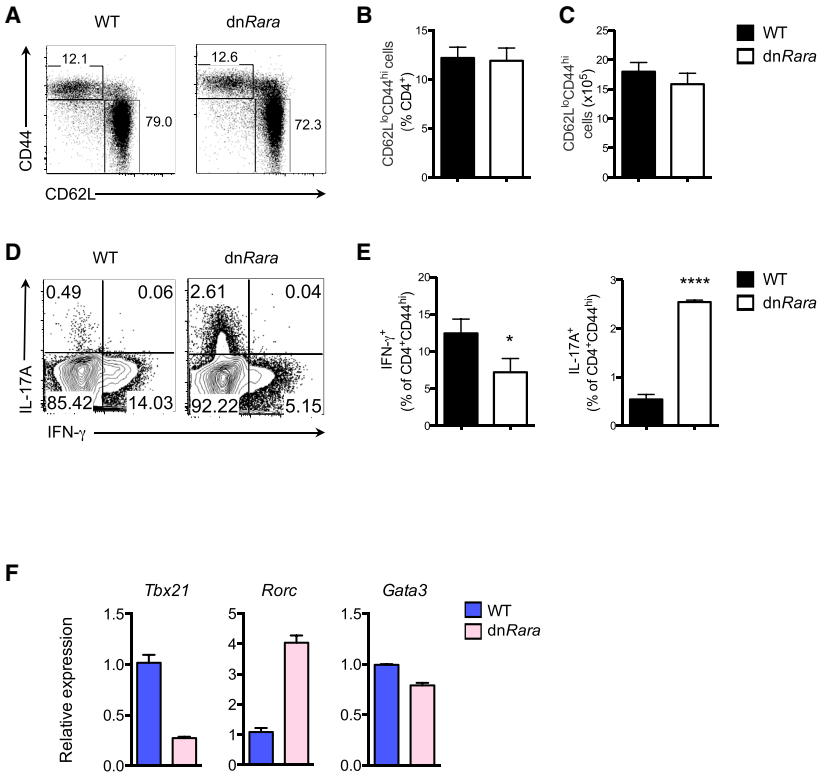

Figure 1. RA Controls the Balance between Th1 and Th17 Effector Cells

(A) Splenic CD4$^+$ T cells from dn*Rara* and WT littermate control mice. Numbers indicate percentage CD62L^{lo}CD44^{hi} cells (top left) or CD62L^{hi} CD44^{lo} T cells (bottom right) gated on CD4$^+$ cells.

(B) Frequency and total number (C) of CD62L^{lo}CD44^{hi} in the CD4$^+$ T-cell population in WT and dn*Rara* mice (n = 3 or 4 per group).

(D) Intracellular IFN-γ and IL-17A expression in splenic CD4$^+$CD44^{hi} T cells after stimulation with phorbol 12-myristate 13-acetate (PMA) and ionoymycin.

(E) Statistical data from cells as in (D).

(F) Quantitative real-time PCR analysis of *Tbx21*, *Rorc*, and *Gata3* in splenic CD4$^+$CD62^{lo}CD44^{hi} cells (as in 1A), sorted by flow cytometry.

Data are from two or three independent experiments with similar results. Mean ± SEM, *p < 0.05; ****p < 0.0001

See also Figure S1.

dose dependent: physiological concentrations of RA enhance Th17-cell differentiation in vitro (Takahashi et al., 2012), yet administration of higher concentrations of RA both in vitro and in vivo negatively regulates Th17-cell responses (Mucida et al., 2007; Takahashi et al., 2012; Xiao et al., 2008). Although RARα has been identified as the critical mediator of RA actions in CD4$^+$ T cells (Hall et al., 2011), to date a comprehensive analysis of the transcriptional targets of RARα in CD4$^+$ T cells has not been reported and the mechanism by which RA regulates these distinct Th-cell fates remains unresolved.

Here we show that RA-RARα is critical for maintenance of the Th1-cell lineage. Loss of RA signaling in Th1 cells resulted in the emergence of hybrid Th1-Th17 and Th17 effector cells. Global analysis of RARα binding and enhancer mapping revealed that RA-RARα directly regulated enhancer activity at Th1-cell-lineage-defining genes while repressing genes that regulate Th17-cell fate. In the absence of RA signaling, infectious and oral antigen induced inflammation resulted in impaired Th1-cell responses with deviation toward a Th17-cell phenotype. These findings identify RA-RARα as a key regulatory node that acts to sustain the Th1-cell response while repressing Th17-cell fate.

RESULTS

RA-RARα Regulates the Balance between Th1 and Th17 Cells

To directly assess the role of RA in Th-cell differentiation in vivo we used mice carrying a sequence encoding a dominant-negative form of the RA receptor RARα (RARα403) targeted to ROSA26 downstream of a *lox*P-flanked "stop" (lsl) cassette. As shown previously (Pino-Lagos et al., 2011), interbreeding with mice expressing Cre recombinase from the *Cd4* promoter generates *Cd4*^{cre}dn*Rara*$^{lsl/lsl}$ progeny (dn*Rara* mice) in which RA signaling is abrogated within the T-cell compartment. In contrast to *Rara*$^{-/-}$ mice, expression of this dnRARα disrupts the RA dependent activity of RARα while retaining the ligand independent effects, allowing the specific analysis of RA-dependent functions.

To investigate the role of RA in the generation of Th-cell subsets under steady-state conditions, we determined the expression of cytokines within CD4$^+$ T cells with an activated CD44^{hi} phenotype. Examination of the peripheral CD4$^+$ T-cell compartment revealed equivalent frequencies and absolute numbers of CD44^{hi}CD62^{lo}CD4$^+$ memory cells in 8-week-old dn*Rara* mice and in Cre$^-$, wild-type (WT), littermate controls (Figures 1A–1C). dn*Rara* effector cells displayed reduced production of IFN-γ compared to their WT counterparts with a >5-fold increase in the frequency of IL-17$^+$ cells (Figures 1D and 1E). Examination of transcripts for the signature lineage-determining TFs showed reduced mRNA expression of *Tbx21* and significantly higher expression of *Rorc* in dn*Rara* effector CD4$^+$ T cells (Figure 1F). Loss of RA signaling had no impact on Th2 effectors with equivalent levels of *Gata3* expression between dn*Rara* and WT mice (Figure 1F) and similar frequencies of IL-4 producing CD4$^+$ T cells (data not shown).

The frequency and numbers of Foxp3$^+$ T cells in the periphery and thymus of dn*Rara* mice were similar to control mice (Figures S1A and S1B), indicating that the increase in Th17 cells was not a consequence of reciprocal regulation by RA of Foxp3$^+$CD4$^+$ T cells and Th17 cells (Mucida et al., 2007). Therefore it is likely that under steady-state conditions RA is critical for differentiation of Th1 cells, while also limiting the differentiation of Th17 cells.

RA Promotes Th1-Cell Differentiation and Inhibits Development of Th17 Cells from Th1 Cell Precursors

We considered two alternative explanations of why dn*Rara* mice exhibit reduced memory effector Th1 cells, in parallel with enhanced Th17 cells. The first possibility was that RA is required for the development of Th1 cells while independently suppressing the primary differentiation of Th17 cells. The alternative possibility was that RA is critical in restraining conversion of Th1 cells to Th17 cells. In order to resolve these two possibilities, naive $CD4^+$ T cells were differentiated in the presence of Th1 or Th17 polarizing cytokines. dn*Rara* expressing $CD4^+$ T cells differentiated under Th1 cell conditions showed a markedly reduced capacity for IFN-γ production (Figure 2A). Diminished cytokine production was not a consequence of impaired proliferative responses as naive $CD4^+$ T cells differentiated under Th1-cell conditions showed robust proliferation, equivalent to WT cells (Figure S2A). In addition, upregulation of the activation markers CD25 and CD44 indicated that dn*Rara* T cells were not impaired in their ability to differentiate into effector cells (Figure S2B). Analysis of TF expression showed that ablating RA signaling resulted in a dramatic reduction in the expression of T-bet in $CD4^+$ T cells differentiated under Th1-cell conditions (Figure 2B). Strikingly, a substantial proportion of dn*Rara* Th1 cells expressed RORγt and co-expression of T-bet and RORγt was observed at the single-cell level. Although we did not observe intracellular IL-17A in cells following brief stimulation with phorbol myristate (PMA) and ionomycin, analysis of supernatants from Th1 polarized cells, reactivated on day 6 of culture on anti-CD3 and anti-CD28 coated plates for 24 hr in non-polarizing media, showed increased expression of IL-17A alongside other Th17-cell-associated cytokines (IL-21 and IL-22) (Figure 2C). Furthermore, mRNA analysis of dn*Rara* Th1 polarized cells revealed dramatic increases in expression of key signature Th17-cell genes (Figure 2D). Notably, these Th1 cells displayed the hallmarks of pathogenic Th17 cells with high amounts of *Il23r* expression but reduced amounts of IL-10 mRNA and protein (Figures 2C and 2D) (Basu et al., 2013).

In order to assess whether enhanced Th17 responses were a general feature of $CD4^+$ T cells in which RA signaling is disrupted, naive $CD4^+$ T cells from dn*Rara* mice were differentiated under Th17 polarizing conditions. In contrast to our observations above, we did not observe an increase in the frequency of IL-17^+ cells in dn*Rara* mice during primary differentiation into Th17 cells (Figure S2C), suggesting that RA restrains Th17-cell differentiation only in the context of a Th1 polarizing cytokine milieu. In support of this, RORγt expression was not observed in dn*Rara* expressing naive $CD4^+$ T cells differentiated under Th0 or Th2 conditions (Figure S2D).

The simultaneous expression of RORγt and T-bet in dn*Rara* Th1 cells suggested that RA-RARα might act to constrain the deviation of Th1 committed cells toward the Th17-cell lineage. To determine whether the RORγt^+ cells represented a distinct T-cell population that arose directly from naive $CD4^+$ T cells or from previously committed Th1 cells, we interbred *Ifng*^{eYFP} (Great) reporter mice with the dn*Rara* mice to allow the tracking of IFN-γ^+ cells. Naive $CD4^+$ T cells from dn*Rara*-*Ifng*^{eYFP} or littermate control mice were activated under Th1 polarizing conditions. On day 7 of culture, $eYFP^+$ (IFN-γ^+) cells were FACS sorted and underwent genome-wide expression analysis. Key signature Th17-cell genes, including Th17-cell cytokines and receptors for cytokines that promote Th17-cell differentiation (*Il17f*, *Il21*, *Il1r1*, *Il6ra*, and *Il23r*), were highly expressed in dn*Rara* IFN-γ expressing cells relative to WT mice, confirming a hybrid Th1-Th17-cell phenotype (Figure 2E). Of note, these Th1-Th17 cells retained high expression of *Il12rb2* and *Cxcr3* mRNA, equivalent to WT Th1 cells, while also expressing *Il23r* (Figure S2E). Genes associated with the Th2-cell subset such as *Gata3* and *Il4* were also dysregulated in dn*Rara* Th1 cells consistent with a role for T-bet in repression of GATA3 (Zhu et al., 2012). These findings show that, in the absence of RA signaling, committed Th1-cell precursors can give rise to cells with a Th17-cell expression signature providing a new perspective on the origins of Th1-Th17 cells. Collectively, these data demonstrate that RA is not only required for Th1-cell differentiation but is also critical in suppressing Th17-cell development in Th1 polarized cells.

RA-RARα Is Required for Late-Phase, STAT4-Dependent T-bet Expression in Th1 Cells

Early expression of T-bet following TCR activation is dependent on IFN-γ, whereas late expression of T-bet (post-termination of TCR signaling) has been shown to be dependent on IL-12 (Schulz et al., 2009). To distinguish a requirement for RA signaling in Th1-cell commitment from maintenance of Th1-cell fate, we examined the kinetics of T-bet expression in naive $CD4^+$ T cells cultured under Th1 polarizing conditions. Induction of T-bet was observed with comparable amounts of T-bet expression between WT and dn*Rara* T cells at day 3 of culture, indicating that RA-RARα signaling is not required for early Th1 lineage commitment (Figure 3A). However, T-bet expression was not sustained in dn*Rara* Th1 cells, with substantially diminished expression of T-bet by day 5 of culture. Given that IFN-γ promotes T-bet expression, the expression of T-bet was examined in the presence of recombinant IFN-γ, in order to avoid potential indirect effects caused by reduced IFN-γ production in dn*Rara* Th1 cells. Exogenous IFN-γ enhanced early T-bet expression in both dn*Rara* and WT Th1 cells but did not rescue the late (>72 hr) impairment in T-bet expression (Figure 3A). IFN-γ signaling, as measured by STAT1 phosphorylation, was not impaired at either time point (data not shown).

The late IL-12-dependent peak of T-bet expression observed in the presence of blocking IFN-γ antibodies was abrogated in dn*Rara* Th1-cell polarized cells (Figure 3A) suggesting impaired STAT4 activity. At day 3 of culture, comparable amounts of phosphorylated STAT4 (pSTAT4) were observed between dn*Rara* and WT mice. By contrast, at day 6 of culture, IL-12 induced pSTAT4 was markedly impaired in dn*Rara* T cells (Figure 3B) despite comparable expression of IL-12Rβ2 mRNA and protein expression and increased expression of *Il12rb1* mRNA (Figure 3C and 3D). Analysis of *Stat4* expression, demonstrated impaired induction of *Stat4* in the absence of RA signaling (Figure 3E) with reduced amounts of total STAT4 protein (Figure 3F). These findings suggest that the observed reduction in pSTAT4 in dn*Rara* Th1 cells is a consequence of diminished STAT4 expression. Consistent with deviation toward the Th17-cell lineage, we observed enhanced pSTAT3 activity in Th1-cell polarized dn*Rara* cells with an increased ratio of pSTAT3/pSTAT4 (Figures S3A and S3B).

To evaluate whether the impairment in T-bet and STAT4 expression correlated with changes in IFN-γ, the time course

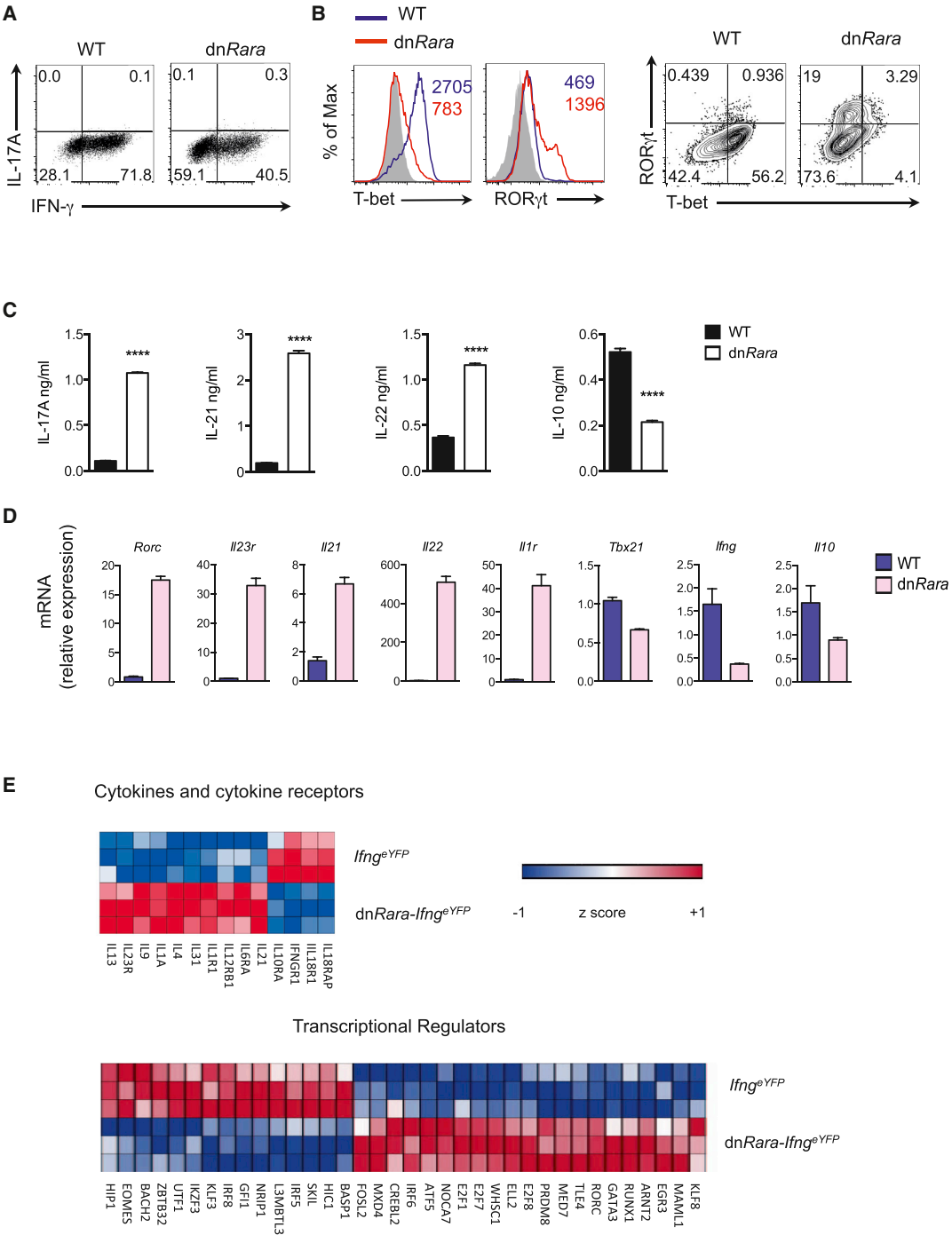

Figure 2. RA Signaling Required for Th1-Cell Differentiation and Repression of Th17-cell Fate in Th1-Cell Precursors

Sorted naive CD4+ T cells from dn*Rara* or WT mice were cultured under Th1 conditions for 6 days.

(A) Intracellular expression of IFN-γ and IL-17A following stimulation with PMA and ionomycin.

(B) T-bet and RORγt expression. Grey histograms indicate staining for *Tbx21*$^{-/-}$ (left panel) or isotype control antibody (right panel). Numbers show MFI. Numbers in quadrants represent percent cells in each.

C) Amount of IL-17A, IL-21, IL-22, and IL-10 in supernatants following restimulation of cells as in (A) with α-CD3 and α-CD28 for 24 hr as measured by multiplex bead array. Triplicate culture wells.

(D) Quantitative real time PCR analysis of Th1 and Th17-cell signature cytokine and TF genes following stimulation with PMA and ionomycin.

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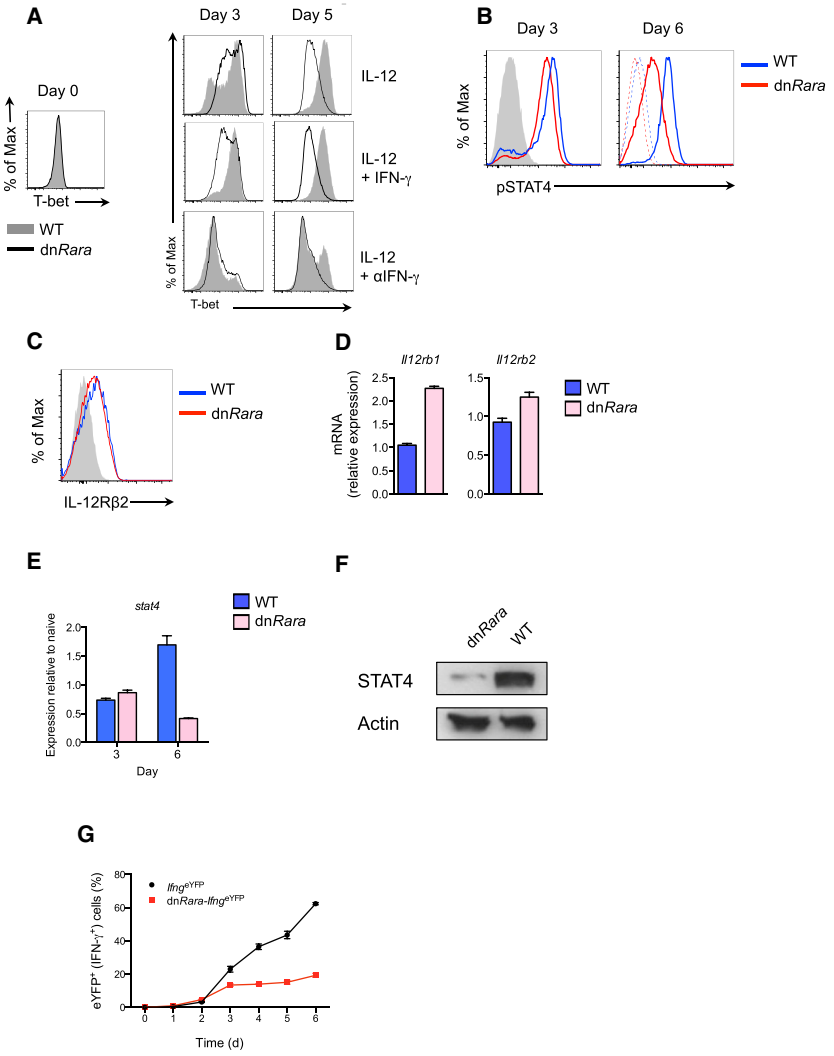


Figure 3. RA Required for Late Phase T-bet Expression

(A) Naive CD4$^+$ T cells from dn*Rara* and WT mice were differentiated under Th1 conditions with combinations of IFN-γ or IFN-γ antibody. T-bet expression analyzed at the indicated time points. Histograms gated on CD4$^+$ T cells.

(B) Flow cytometric analysis of STAT4 phosphorylation in naive CD4$^+$ T cells from dn*Rara* and WT mice differentiated under Th1 conditions. Cells analyzed directly from culture after 3 days (left panel) or on day 6 following treatment with (solid lines) or without (dashed lines) 25 ng/ml IL-12 for 30 min (right panel). Shaded histogram displays pSTAT4 staining in cells cultured under Th0 conditions.

(C) Cell-surface expression of IL-12Rβ2 on day 6 of culture.

(D) Quantitative real-time PCR analysis of *Il12rb1 and Il12rb2* on day 6.

(E) Quantitative real-time PCR analysis of *Stat4* in Th1 polarized cells at indicated time points. Expression relative to naive CD4$^+$ T cells.

(F) Western blot analysis of total STAT4 protein on day 6 of Th1 culture.

(G) Naive CD4$^+$ T cells from dn*Rara*-*Ifng*^{eYFP} and control mice were activated under Th1 conditions. Frequency of IFN-γ$^+$ (eYFP$^+$) cells at indicated time points, gated on viable CD4$^+$.

Data representative of two to three independent experiments. Mean ± SEM.

See also Figure S3.

of IFN-γ expression following initiation of Th1-cell polarization was analyzed in naive dn*Rara*-*Ifng*^{eYFP} expressing CD4$^+$ T cells. The kinetics of IFN-γ induction, as measured by frequency of eYFP$^+$ cells, closely mirrored WT cells during the first 72 hr of culture but expression was not sustained in the absence of RA signaling (Figure 3G). Collectively, these data show that RA plays a temporal role in Th1 differentiation, maintaining Th1-cell commitment through regulation of T-bet and STAT4.

RA-RARα Regulates Th1-Cell Plasticity

Alterations in the stable expression of lineage-determining TFs are thought to underlie Th-cell stability or plasticity. The emergence of Th1-Th17 cells together with the loss of T-bet expression, suggested a role for RA in the regulation of Th1-cell plasticity. However, diminished T-bet and STAT4 activity from day 3 of primary Th1-cell differentiation prevented assessment of lineage stability in fully differentiated Th1 cells. To determine whether RA-RARα was required for long-term Th1-cell fate, we differentiated naive CD4$^+$ T cells from dn*Rara*$^{lsl/lsl}$ mice under Th1-cell conditions, treated them with TAT-Cre (Wadia et al., 2004) on days 5 and 7, and restimulated them under Th1-cell conditions for a further 5 days. The temporal loss of RA signaling in Th1 cells resulted in decreased T-bet expression with a reciprocal increase in RORγt expression (Figure 4A). ~50% of cells expressed RORγt, which suggests that ongoing RA-RARα activity is critical for sustaining T-bet and suppressing Th17-cell fate. Alterations in the lineage determining TFs did not impact on the cytokine phenotype (Figure S4A). This might in part reflect T-bet independent regulation of the *Ifng* locus at late stages in Th1-cell development.

To further examine the role of RA in Th1-cell stability, naive CD4$^+$ T cells from *Ifng*^{eYFP} mice were differentiated under

(E) Naive CD4$^+$ T cells from dn*Rara*-*Ifng*^{eYFP} and *Ifng*^{eYFP} mice were cultured under Th1 conditions. IFN-γ (eYFP$^+$) cells were sorted on day 7 following stimulation with PMA and ionomycin. Heatmaps displaying the fold changes of genes that were differentially expressed (fold change > 1.5, $p < 0.05$) for selected cytokines or cytokine receptors (upper panel) and TFs (lower panel). Samples from three independent experiments.

Representative data of at least three (A and B) or two (C and D) independent experiments. Mean ± SEM.

See also Figure S2.

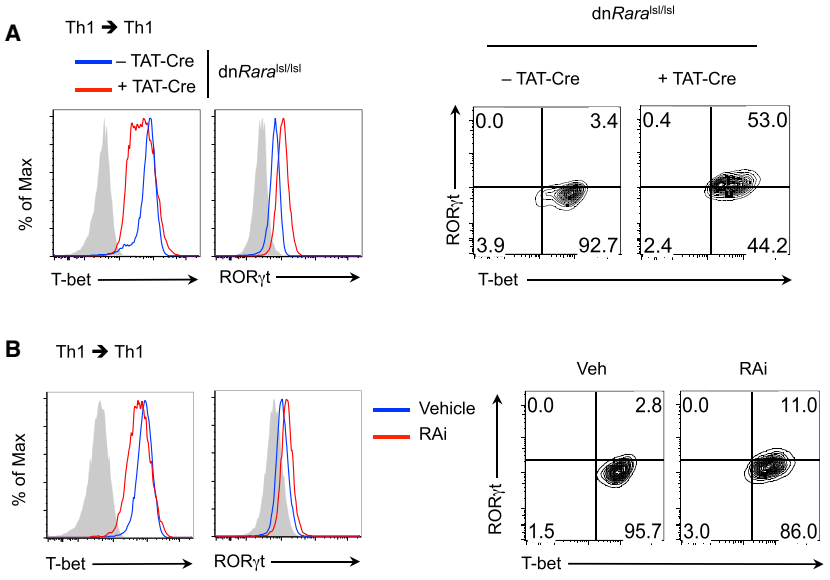


Figure 4. Loss of RA Signaling in Fully Committed Th1 Cells Leads to Th1 Plasticity and Divergence Toward the Th17 Lineage

(A) Naive CD4$^+$ T cells from dn*Rara*$^{lsl/lsl}$ mice were differentiated under Th1 conditions. Th1 cells were transduced with TAT-Cre on days 5 and 7 and re-polarized under Th1 conditions for a further 5 days. Intracellular expression of T-bet and RORγt.

(B) Naive CD4$^+$ T cells from *Ifng*^{eYFP} mice were differentiated under Th1 conditions. IFN-γ (eYFP$^+$) cells were sorted on day 7 and restimulated under Th1 conditions for 5 days in the presence of Veh or RAi. Intracellular expression of T-bet and RORγt. Data representative of two independent experiments.

See also Figure S4.

Th1-cell polarizing conditions. eYFP$^+$ (IFN-γ$^+$) cells were FACS-sorted on day 7 of culture and restimulated under Th1-cell conditions in the presence of the RAR inhibitor LE540 (RAi) or vehicle control (Veh). Inhibition of RA signaling in fully committed Th1 cells propagated for a further 5 days under Th1 conditions resulted in downregulation of T-bet and the emergence of cells co-expressing RORγt (Figure 4B). Diminished T-bet expression was associated with modest reductions in IFN-γ expression (Figure S4B). Taken together, these data establish that loss of RA signaling in fully committed Th1 cells leads to transdifferentiation to progeny with features of the Th17 lineage and support a model where RA constrains late-stage plasticity of Th1 cells.

RA-RARα Regulates Enhancer Activity at Lineage Determining Th1-Cell Genes

To better understand the molecular mechanism by which RARα regulates Th-cell fate, we performed genome-wide analysis of RARα binding in WT Th1 cells by ChIP-Seq, combined with transcriptional profiling of dn*Rara*-expressing Th1 cells in order to identify functional targets of RARα. Selected loci were validated by ChIP-qPCR. RARα binding was identified at 1,766 sites in 1,567 genes. RARα binding was detected at 10.3% (76 of 740 genes) of genes downregulated in the absence of RA signaling (Table S2) (hereafter referred to as positively regulated) and 4.8% (56 of 1,169) of the upregulated genes (Table S3). In keeping with its classical role as a positive regulator of transcriptional activation there was significant enrichment of RARα binding at genes positively regulated by RA (Fisher's exact test, $p < 0.0001$). However, the presence of RARα at a subset of the negatively regulated genes indicates that RA-RARα also plays a role in transcriptional repression within Th1 cells.

RA-RARα-dependent loci included Th1-cell lineage-defining genes (*Tbx21* and *Stat4-Stat1*). In addition to targeting the *Tbx21* promoter (Figures 5A and 5B), modest RARα binding was observed at the conserved T-bet enhancer element, 12kb upstream of the transcriptional start site (TSS) (Yang et al., 2007). This was confirmed by ChIP-qPCR (Figure 5B). Intergenic RARα was also detected at the *Stat4-Stat1* locus and an *Ifng* enhancer element (Figures S5A and S5B).

RA binding to nuclear RARα results in recruitment of co-activator complexes containing the histone acetyl-transferases p300 and CBP (Kamei et al., 1996). p300 is highly enriched at enhancer regions where it acetylates H3K27, a marker of active enhancers (Rada-Iglesias et al., 2011), suggesting a possible role for RA-RARα in regulating enhancer activity. To test this, we mapped genome-wide binding of p300, H3K4me1, H3K4me3, and H3K27ac histone modifications in dn*Rara* and WT Th1-cells, validating selected regions by ChIP q-PCR. Active enhancers were operationally defined as regions with increased intensity of H3K4me1, p300, and H3K27ac with low or absent H3K4me3 (Rada-Iglesias et al., 2011).

RARα binding at the *Tbx21*, *Stat4*, and *Ifng* loci co-localized with p300 binding at enhancer regions (Figures 5A and S5A). dnRARα lacks the activation function 2 (AF2) domain which is required for RA-dependent recruitment of coactivators. Consistent with this, dn*Rara* expressing T cells exhibited a significant reduction in p300 occupancy and H3K27ac deposition at the *Tbx21* enhancer, supporting the direct regulation of enhancer activity by RA-RARα (Figures 5A and 5C). p300 binding at the *Ifng* and putative *Stat4* intergenic enhancers was also dependent on RA-RARα (Figures S5A and S5C). Loss of p300 binding at the *Stat4-Stat1* intergenic enhancer in *dnRara* Th1 cells correlated with reduced *Stat4* transcripts, whereas *Stat1* expression was actually increased, suggesting that this enhancer element regulated *Stat4* transcription. A recent study identified a role for STAT4 in the regulation of Th1 enhancers (Vahedi et al., 2012). Given that STAT4 expression was reduced in dn*Rara* Th1 cells, it was possible that the loss of p300 was in part due to reduced expression of STAT4. To address this issue, we assessed the binding of STAT4 in WT Th1 cells and compared p300 occupancy in WT and *Stat4*$^{-/-}$ Th1 cells using publicly available ChIP-seq data (Table S1) (Vahedi et al., 2012; Wei et al., 2010). Although STAT4 binding was observed at the *Tbx21* enhancer, loss of STAT4 was not associated with obvious differences in p300 binding (Figure S5D), arguing for a direct contribution of RARα to p300 recruitment and enhancer activity. Collectively, these data show that RA regulates expression of key Th1-cell lineage genes through remodeling of enhancer regions.

RA-RARα Represses Th17-Cell Fate in Th1 Cells through Direct Regulation of Th17-Cell Genes

The earlier finding that Th1 cells acquired features of Th17 cells in the absence of RA signaling led us to evaluate direct regulation of Th17-cell-instructing genes by RA-RARα. We first investigated effects of RA on the Th17-cell pioneer factors BATF and IRF4. As previously reported (Basu et al., 2013), these genes were expressed in WT Th1 cells. Strikingly, kinetic analysis of *Batf* and *Irf4* expression in naive cells stimulated under Th1-cell conditions revealed dramatic upregulation of IRF4 (40- to 60-fold) during the initial phase of Th1-cell polarization with comparable expression between dn*Rara* and WT cells (Figure 5D). Loss of RA signaling resulted in derepression of BATF-IRF4 target genes, *Rorc*, *Il23r*, *Il22*, *Il21*, and *Il12rb1* (Figure 5E). This suggested that "balancing" factors must be induced in an RA-dependent manner to restrict the actions of BATF-IRF4 complexes at Th17-cell genes. IRF8, an alternative binding partner for BATF, previously shown to suppress Th17 differentiation (Ouyang et al., 2011), was one of the RARα target genes most suppressed in dn*Rara* Th1 cells. In WT Th1 cells, induction of *Irf*8 expression paralleled *Irf4* expression. However, in dnRara cells *Irf8* expression was not sustained past 24 hr (Figure 5D). RARα bound at a putative upstream enhancer (Figures 5F and 5G) and in the absence of RA signaling, reduced p300 and H3K27ac were observed at this locus (Figure 5H and 5I). Together, these data show that RA directly regulates expression of IRF8 in Th1 differentiating cells and suggests a potential mechanism by which BATF-IRF4 activity is constrained within early Th1 cells.

Transcriptional activation of BATF-IRF4 target genes is dependent on STAT3 and RORγt (Ciofani et al., 2012). Various genes for cytokines and cytokine receptors associated with STAT3 activation (*Il21*, *Il1r1*, *Il6ra*, and *Il23r*) were derepressed in dn*Rara* Th1 cells (Figure 5E). RARα targeted the promoter and an upstream enhancer in the *Il6ra* locus (Figure 5G) with increased H3k27ac observed at the enhancer element in dn*Rara* Th1 cells (Figure 5J). Consistent with this, dn*Rara* Th1 cells failed to downregulate mRNA and cell-surface IL6-Rα expression during Th1 polarization (Figures S5E and S5F). These findings suggest that RA regulates Th1-cell plasticity in part by inhibiting responsiveness to IL-6.

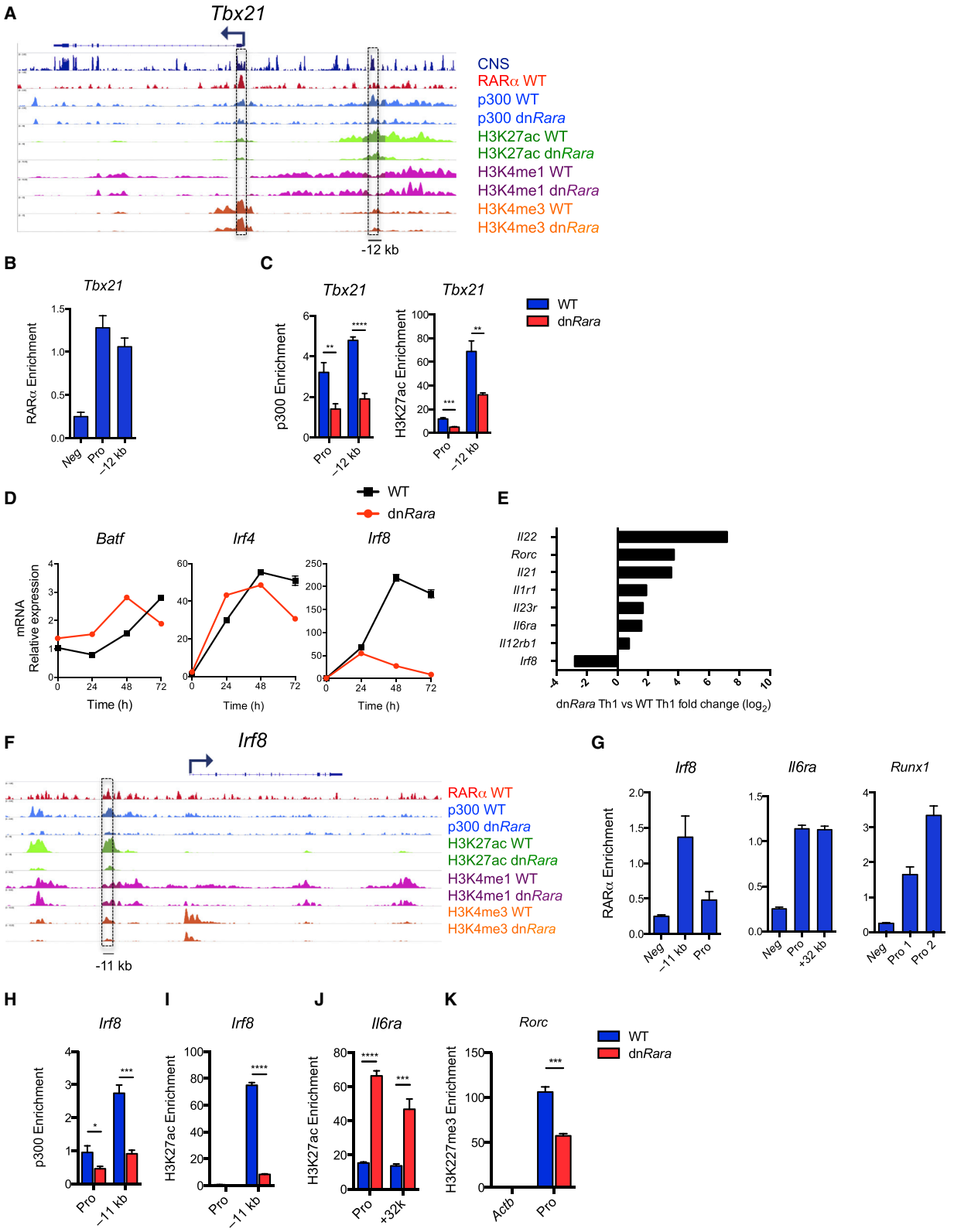
RORγt was not a direct target of RARα. However, disruption of RA signaling resulted in increased expression of *Runx1*, a TF associated with transactivation of *Rorc* (Figure S5E) (Zhang et al., 2008). ChIP analysis confirmed direct regulation of short and long *Runx1* isoform promoters by RA-RARα (Figure 5G). In Th1 cells, the *Rorc* locus is epigenetically silenced by T-bet (Mukasa et al., 2010). However, in dn*Rara* cells, the repressive H3K27me3 mark was reduced at RORγt isoform-specific exon (Figure 5K), consistent with loss of T-bet. These findings suggest that increased RORγt expression in the absence of RARα signaling is in part due to increased accessibility of the *Rorc* locus, with unrestrained activation by Runx1. Collectively these data indicate that RA-RARα antagonises the activity of the core Th17-cell instructing TFs (IRF4, BATF, STAT3, and RORγt), both directly and indirectly, to suppress the Th17-cell gene program. Notably, Th2-cell-associated genes were not identified as targets of RARα (Tables S2 and S3) suggesting that direct repression of alternative cell fates by RA-RARα is specific to the Th17-cell program.

Th1-like Th17 Cells Emerge during Infection with *L. monocytogenes* in the Absence of RA Signaling

To assess the significance of these findings for immune responses in vivo, we intravenously infected WT and dn*Rara* mice with an attenuated strain of *L. monocytogenes* (ΔActA), Lm-2W, which allows tracking of $CD4^+$ T cells specific for listeriolysin O peptide $LLO_{190–201}$ (LLOp). At the peak of the response, $CD4^+$ T cells were isolated from the spleen and LLOp antigen-specific T cells were assayed for expression of cytokines and the TFs, T-bet, and RORγt. dn*Rara* mice mounted an effector-T-cell response of similar magnitude to WT mice with comparable frequencies and total numbers of $CD44^{hi}$LLOp:I-A^b-specific $CD4^+$ T cells (Figures 6A and 6B). In WT mice, Lm-2W induced a Th1-cell restricted response, as evidenced by high T-bet expression within the LLOp-specific T-cell fraction (Figure 6C). LLOp:I-A^{b+} CD4+ T cells from dn*Rara* mice expressed lower amounts of T-bet and a substantial proportion expressed RORγt, with co-expression of these TFs observed in a subset of cells (Figure 6C). At day 7 post-infection, a significant proportion of $CD4^+$ T cells isolated from the spleen of dn*Rara* mice were IL-17^+ or dual IL-$17A^+$IFN-γ^+ with a trend toward reduced frequency of IFN-γ^+ cells (Figure 6D). Measurement of cytokine protein concentrations from splenocytes restimulated with LLOp confirmed reduced amounts of IFN-γ and concomitant increase in IL-17A (Figure S6A). We did not detect IL-4 production by intracellular staining or protein secretion (Figure S6A and S6B). Consistent with our in vitro data showing downregulation of IL6-Rα on WT Th1 cells, cell-surface IL-6Rα was not detectable on WT LLOp:I-A^{b+} $CD4^+$ T cells. However, dn*Rara* LLOp:I-A^{b+} $CD4^+$ T cells retained expression of IL-6Rα (Figure S6C), supporting a potential role for IL-6 signaling in the regulation of Th1-cell plasticity. These findings establish that RA-RARα signaling in T cells constrains the emergence of Th17 cells in a Th1-cell-instructing microenvironment in vivo.

RA Regulates the Th1-Th17-Cell Axis in the Gut and Prevents the Development of Intestinal Inflammation

RA is constitutively synthesized by a subset of DCs in the gut. To address the physiological importance of RA signaling in the regulation of pathogenic intestinal $CD4^+$ T cells, we interbred dn*Rara* mice with OTII mice that transgenically express an ovalbumin (OVA)-specific TCR and transferred naive $CD4^+$ T cells from OTII(dn*Rara*) or WT OTII mice into *Rag1*$^{-/-}$ hosts. Recipients were maintained on an OVA-containing diet for 7 days to induce differentiation within the transferred cells and migration to the intestinal tissue. Consistent with the infection experiments, feeding OTII(dn*Rara*)-recipient mice OVA resulted in a shift in the Th1-Th17-cell balance with a deficiency in IFN-γ-producing cells and increased frequency of IL-17^+ and dual IFN-γ^+IL-17^+ cells in the mesenteric lymph node (MLN), lamina propria lymphocytes (LPL), and spleen (Sp), 7 days after transfer (Figures 7B and 7C). To address the functional significance of the dysregulated cytokine response in dn*Rara* T cells, we orally challenged mice with OVA and evaluated them for development of intestinal inflammation and diarrhea (Figure 7A). Recipients of OTII(dn*Rara*) cells developed accelerated wasting disease relative to mice that received WT OTII cells (Figure 7D). Whereas all of the recipients of OTII(dn*Rara*) cells developed severe diarrhea



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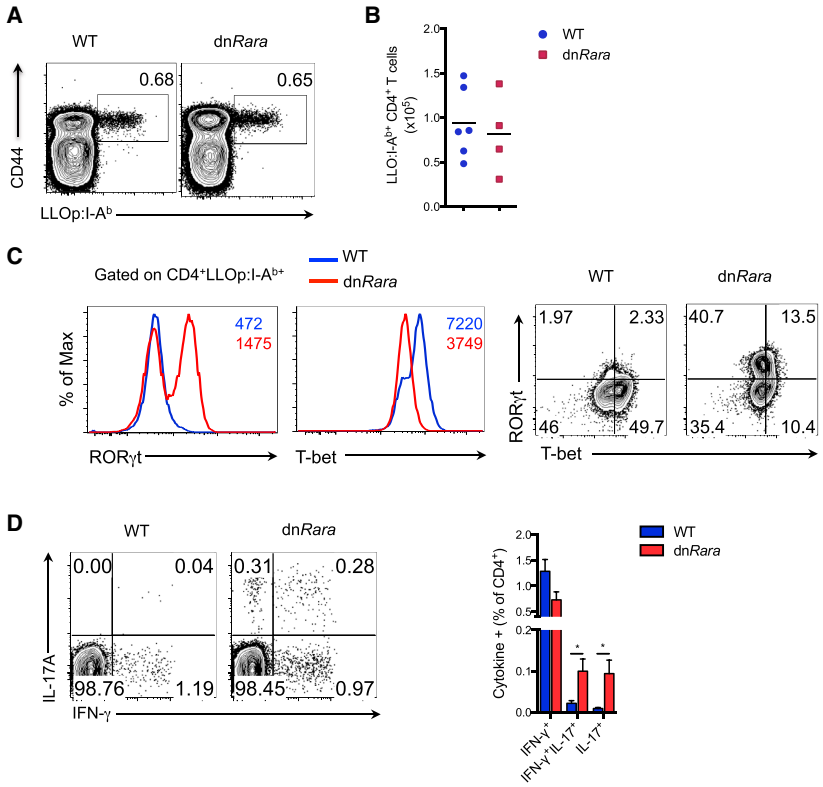

Figure 6. RA Signaling Required to Prevent the Generation of Th17 Cells during Infection with *L. monocytogenes*

(A) Frequency of LLOp:I-A^b CD4$^+$ T cells isolated from spleen of dn*Rara* and WT mice 7 days after infection with an attenuated strain of *L. monocytogenes* (Lm-2W). Gated on CD4$^+$ T cells.

(B) Absolute numbers of LLOp:I-A^b CD4$^+$ T cells as in (A).

(C) Intracellular T-bet and RORγt expression gated on LLOp:I-A^b CD4$^+$ T cells.

(D) Intracellular staining for IFN-γ and IL-17A following stimulation of splenocytes with LLOp for 6 hr, 7 days after infection with Lm-2W. Gated on CD4$^+$ T cells. Right panel shows statistical data pooled from three independent experiments (3–6 mice per group).

Representative data of at least three (A and B), or two independent experiments (C). Mean ± SEM. See also Figure S6.

by day 12 (Figure 7E), recipients of WT cells remained diarrhea free. Cytokine production was also assessed after the first gavage and confirmed an increased frequency of IL-17$^+$ cells with concomitant reduction in IFN-γ$^+$ cells. Notably, enhanced IL-17 responses were not a consequence of impaired Foxp3$^+$ conversion (Figure 7F). Homing of transferred cells to the gut was not affected in this model with similar frequencies of CD4$^+$ T cells detected in the gut tissues (Figure S7A). We conclude that loss of RA signaling leads to deviation from Th1 to Th17 phenotype both in the periphery and the gut where these Th17 cells are associated with significant intestinal inflammation.

DISCUSSION

Dysregulated Th-cell responses underlie the pathogenesis of autoimmune and allergic disease. In contrast to T regulatory (Treg) cells and Th17 cells, the Th1-cell lineage is thought to be relatively stable. However, the factors that control maintenance of the Th1-cell lineage were not previously known. This study identifies RA-RARα as a central regulatory node in the transcriptional network governing Th1-cell stability. We found that RA-RARα directly sustained the expression of lineage determining Th1-cell-associated genes during naive T-cell differentiation while also repressing signature Th17-cell-associated genes. Ablation of RA signaling in Th1-committed cells resulted in enhanced Th1-cell plasticity with deviation towards a Th17-cell phenotype. Using ChIP-seq to identify regulatory elements, we found that RARα bound at enhancers and recruitment of p300 to these regions was dependent on RA signaling. In vivo, both Th17 and Th1-Th17 cells emerged during infection with *L. monocytogenes* and in a model

Figure 5. RA-RARα Regulates Enhancer Activity at Th1 Lineage Associated Loci and Represses Th17 Genes

Naive CD4$^+$ T cells from WT and dn*Rara* mice were cultured for 6 days under Th1 conditions prior to chromatin precipitation and transcriptional profiling.

(A) ChIP-seq binding tracks at *Tbx21* locus for RARα in WT Th1 cells and p300 binding, H3K27ac, H3K4me1, and H3K4me3 modifications in WT and dn*Rara* Th1 cells.

(B) Validation of the RARα-binding regions in WT Th1 cells by ChIP-qPCR. Untr6 region serves as a negative control. Binding events per 1,000 cells displayed as "Enrichment."

(C) The effects of dn*Rara* expression on p300 and H3k27ac abundance at the Tbx21 locus were validated by ChIP-qPCR.

(D) Quantitative real-time PCR analysis of *Batf*, *Irf4*, and *Irf8* mRNA in naive CD4$^+$ T cells from dn*Rara* or WT cells differentiated under Th1-cell conditions for 0, 24, 48, 72 hr. Mean ± SEM, replicate wells.

(E) Log2 values of fold changes in gene expression as measured by microarray analyses. Average fold change depicted.

(F) ChIP-seq binding tracks at *Irf8* locus for cells as in (A).

(G) Validation of RARα ChIP-seq regions by ChIP-qPCR.

(H–J) ChIP analysis of p300 and H3K27ac at selected loci.

(K) ChIP analysis of H3K27me3 at the RORc locus. *Actb* locus serves as a negative control.

Data from three independent experiments (E) or representative of two independent experiments (B–D, G–K); Mean ± SD unless noted otherwise. Abbreviation: pro, promoter.

See also Figure S5.

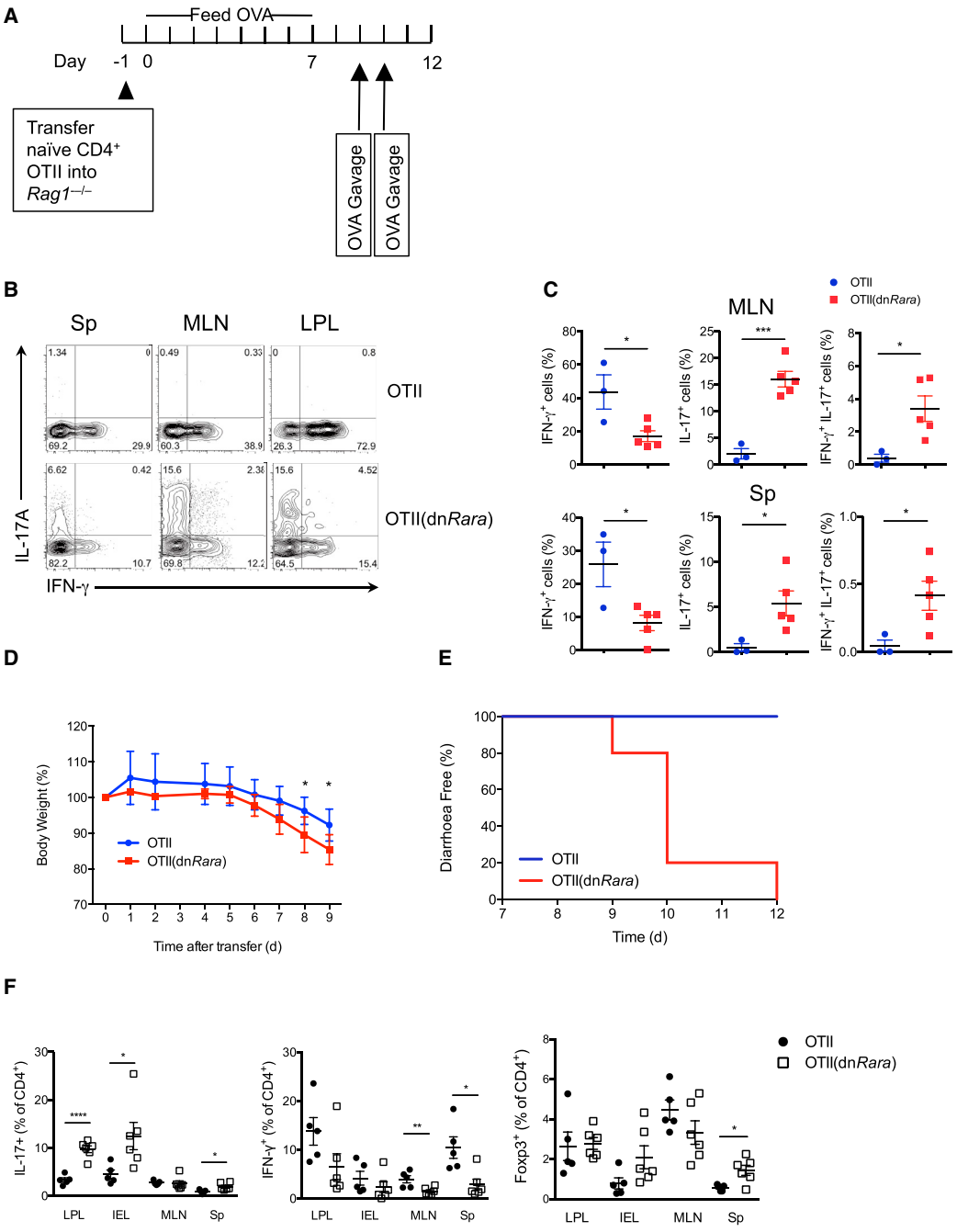

Figure 7. Loss of RA Signaling Causes Dysregulated Th1 and Th17 Response and Increased Pathogenicity in a Model of Gut Inflammation

(A) Schematic illustration of the adoptive transfer experiment.

(B) Intracellular expression of IL-17A and IFN-γ among $CD4^+$ cells from the spleen (Sp), mesenteric lymph nodes (MLN), and lymphocytes from the lamina propria (LPL) of mice as in (A) 7 days after transfer.

(C) Statistical data for frequency of IFN-γ^+, IL-17^+, and IFN-γ^+IL-17^+ cells as in (B) in MLN and Sp.

(D) Percentile change of original body weight in *Rag1*$^{-/-}$ recipients treated as in (A) (n = 5–7 per group). Mean ± SD.

(E) Frequency of diarrhea-free mice among *Rag1*$^{-/-}$ recipients as in (A) (OTII recipients n = 3, OT-II(dn*Rara*) recipients n = 5).

(F) Frequencies of IL-17, IFN-γ, and Foxp3 in $CD4^+$ cells isolated from Sp, MLN, LPL, and IELs of mice as in (A), 9 days after transfer (n = 5 or 6 per group).

Data from one experiment (B and C), pooled from two independent experiments (D and F), or representative of two independent experiments (E). Mean ± SEM unless otherwise noted.

of oral tolerance. In the latter, their presence was associated with significant pathology.

Enhancers play a key role in directing cell fate through the regulation of lineage specifying genes. Enhancer profiling in WT and dn*Rara* T cells revealed RA-dependent activation of enhancers at genes critical for Th1 identity (*Tbx21*, *Stat4*, *Ifng*, and *Irf8*). RA-dependent changes in p300 and H3K27ac were reflected at the transcriptional level suggesting that, in addition to its classical role as a transcriptional regulator, RA regulates gene expression in an enhancer-dependent manner. Although the ability of RA-RARα to target p300-CBP complexes to nucleosomes is well established, regulation of enhancers by RA has not been widely studied. We propose that unliganded RARα at enhancer elements acts as a gatekeeper, enabling initiation of enhancer activation once T cells sense RA in the microenvironment. A similar role has been demonstrated for STAT proteins (Vahedi et al., 2012), suggesting that environmental cues act as checkpoints for initiation of enhancer activation and T-cell fate. Although H3K4me1 modifications are present at early time points during T-cell differentiation, conversion to "active" status requires acquisition of H3K27ac, which is often not evident until later stages of differentiation (Hawkins et al., 2013). Consistent with a temporal role for enhancers in maintenance of gene expression, RA signaling was not required for initiation of transcription of target genes but rather acted to maintain their expression. These data highlight the importance of enhancers in maintenance of cell identity and plasticity. It is possible that RA-RARα regulation of enhancers represent the major mechanism by which RA regulates cell fate. A recent study identified enrichment of RARα at enhancers in embryonic stem cells (Chen et al., 2012). Given that the RA-RARα axis is a highly conserved signaling pathway, which plays a critical role in regulating cell-fate specification during embryogenesis and cell differentiation, it will be important to evaluate a broader role for RA-RARα in regulation of enhancer functionality, both in alternative Th-cell subsets and outside of the immune system.

In addition to sustaining expression of Th1-cell-associated genes, we found that RA actively silences genes implicated in Th17-cell differentiation. Among genes known to regulate the Th17-cell program, *Runx1* and *Il6ra* were directly repressed by RA-RARα. In addition, BATF-IRF4 target genes were derepressed in the absence of RA signaling. In Th17 cells, BATF-IRF4 complexes act co-operatively as pioneer factors at key Th17 genes (Ciofani et al., 2012), modulating chromatin accessibility to facilitate binding of STAT3 and RORγt. On the basis of their expression in alternative Th-cell subsets, it has been suggested that BATF-IRF4 complexes play a universal role in establishing binding of lineage-specific TFs (Ciofani et al., 2012). However, BATF deficiency does not impact on Th1-cell differentiation (Schraml et al., 2009). An alternative model is that upregulation of BATF and IRF4 confers plasticity in early Th1 cells, poising chromatin specifically at Th17-cell-associated genes. IRF8, an alternative binding partner for BATF, negatively regulates Th17-cell differentiation (Ouyang et al., 2011). Our results identified IRF8 as a member of the Th1-cell transcriptional network whose expression was critically dependent on RA signaling. Induction of IRF8 would be expected to limit plasticity of Th1 cells by repressing Th17 differentiation, potentially by competing for binding to BATF. In support of a role for IRF8 in regulation of Th1-Th17 axis, patients with mutations in IRF8 have impaired Th1 responses (Hambleton et al., 2011) and single nucleotide polymorphisms (SNPs) in *Irf8* are associated with several autoimmune diseases in which IFN-γ^+ Th17 cells play a pathogenic role (Franke et al., 2010; Cunninghame Graham et al., 2011). It will be of interest to identify transcriptional targets of BATF, IRF4, and IRF8 in Th1 cells.

RA signaling was critical to maintain appropriate Th1-cell responses and suppress the development of IL-17$^+$ and IFN-γ^+IL17$^+$ cells. Hybrid Th1-Th17 cells are implicated in the pathogenesis of several autoimmune diseases. Their development has been attributed to the plasticity of Th17 cells. Our findings suggest that these cells might alternatively reflect Th1 plasticity and suggest a novel developmental pathway for Th17 cells. Th1 derived "Th17" cells expressed high levels of the receptor for IL-23, a critical determinant of Th17 pathogenicity (Basu et al., 2013), and were associated with significant gut inflammation and pathology in a model of oral tolerance. Further experiments are required to test the prediction that pathogenic Th17 and IFN-γ^+IL-17$^+$ cells which arise in autoimmunity emerge from Th1 cells when RA is deficient or its signaling perturbed.

A range of inflammatory stimuli can induce RA synthesis and signaling during the course of an immune response. Our results suggest that in a Th1-cell instructing microenvironment the dominant action of RA is to repress Th17-cell fate and promote Th1-cell responses. We did not observe enhanced Th17-cell responses during primary Th17-cell differentiation, suggesting that the impact of RA on T-cell stability might vary both temporally and among tissues. Previously we have shown in a model of skin allograft rejection that impaired Th1 responses in dn*Rara* mice were accompanied by increased Th2-cell cytokines (Pino-Lagos et al., 2011). We did not identify direct repression of Th2-cell-associated genes by RARα. However, T-bet suppresses GATA3 (Zhu et al., 2012) and in the presence of a Th2 skewing microenvironment, such as the skin, impaired expression of T-bet in the absence of RA signaling renders cells susceptible to Th2 deviation. Thus, the effects of RA on T-cell fate are likely dependent on external and intrinsic factors that shape T-cell polarity.

In summary, we show that RA signaling plays a critical role in regulating stability and functional plasticity of Th1 cells. Regulation of enhancer activity at lineage determining genes by RA-RARα provides mechanistic evidence for reciprocal regulation of Th1 and Th17-cell programs. In the absence of RA signaling, downmodulation of T-bet, STAT4, and IFN-γ, and loss of repression of Th17-cell genes, creates a permissive environment for transdifferentiation of Th1 cells to Th17 cells. This study identifies the RA-RARα axis as a potential node for intervention in diseases in which dysregulation of the Th1-Th17-cell axis is observed.

EXPERIMENTAL PROCEDURES

Mice

C57Bl/6 dn*Rara* mice have been described previously (Pino-Lagos et al., 2011). *Ifng*^{eYFP} (GREAT) mice were purchased from the Jackson Laboratory. Mice were bred and maintained at Charles River Laboratory, UK, in pathogen-free conditions. All animal experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986. C57Bl/6 OTII(dn*Rara*), OTII, and *Rag1*$^{-/-}$ mice were bred and maintained at the Rockefeller University specific pathogen-free animal facility.

Cell Isolation, Cell Culture, and Flow Cytometry

Sort purified, naive $CD4^+CD25^-CD44^{lo}CD62L^{hi}$ T cells were cultured with T-cell depleted splenocytes (APCs) and anti-CD3 under polarization conditions for Th0, Th1, Th2, and Th17-cell-associated subsets. Details are provided in the Supplemental Experimental Procedures. For analysis of cytokine production, cells were restimulated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin in the presence of monensin for 4–5 hr at 37°C. Cells were stained with LIVE/DEAD Dead Cell Stain (Invitrogen), followed by staining for cell-surface markers and then fixed and permeabilized (BD Biosciences) for staining of intracellular antigens. Flow cytometry was performed on a LSR Fortessa (BD Biosciences) and analyzed with Flowjo software (Tree Star).

TAT-Cre Transduction

Sort purified naive $CD4^+$ T cells were differentiated under Th1 conditions. After 5 days, cells were treated with 50 μg/ml TAT-Cre peptide for 45 min at 37°C, then washed and expanded in IL-2-containing medium. After 48 hr cells were retreated with Tat-Cre followed by polarization under Th1 conditions.

Real-Time Quantitative PCR

Total RNA was extracted from cells with RNeasy Mini kit (QIAGEN) and cDNA was synthesized with Qscript RT kit (Quanta). Quantitative gene-expression analysis was performed using Taqman primer probe sets (Applied Biosystems), listed in Table S4. Expression of target genes was normalized to β-actin.

Microarray Gene-Expression Profiling

For gene-expression analysis Affymetrix (for *Ifng*^{eYFP} dataset) or Agilent (for the dn*Rara* Th1 dataset) microarray chips were used. Differentially expressed genes were detected using fold-change and t test analysis. See Supplemental Experimental Procedures for further information.

Chromatin Immunoprecipitation and ChIP-Seq

Immunoprecipitation and DNA sequencing was performed by Active Motif. The following antibodies were used: anti-H3K27me3 (Millipore 07–449), anti-p300 (Santa Cruz sc–551X), anti-H3K4me1 (Active Motif 39287), anti-H3K4me3 (Active Motive 39159), anti-H3K27ac (active Motif 39133), anti-RARα (Diagenode C15310155). Illumina sequencing libraries were prepared from the ChIP and Input DNAs. For ChIP q-PCR, enrichment calculated as binding events per 1,000 cells using Active Motif's normalization scheme. Detailed methods for ChIP-seq and binding site analyses are provided in the Supplemental Information.

L. monocytogenes Infection

Mice were infected i.v. with 1×10^6 cfu *L. monocytogenes* and spleens were harvested 7 days later. Splenocytes were enriched for $CD4^+$ T cells with a $CD4^+$ T-cell negative selection microbead kit (Miltenyi Biotec) and stained with PE labeled, LLO:I-A^b dextramer (Immudex) and cell-surface antibodies. For analysis of intracellular cytokine production, splenocytes were restimulated with LLO peptide (PiProteomics) at 10 μg/ml for 6 hr.

Food-Antigen-Induced Diarrhea Model

Naive $CD4^+$ T cells from OTII or OTII(dn*Rara*) were intravenously transferred to $Rag1^{-/-}$ mice. These mice were then maintained on a diet containing OVA for 7 days and challenged with oral OVA on days 9 and 10. Lymphocytes were isolated from the intestinal epithelium, lamina propria, MLN, and spleen at the indicated time points after the start of oral OVA exposure of the recipient mice. Detailed experimental procedures are described in the Supplemental Experimental Procedures.

Statistical Analysis

Statistical significance was calculated by unpaired two-tailed Student's t test with Graphpad Prism software. p values < 0.05 were considered significant. p values are denoted in figures as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

ACCESSION NUMBERS

Chip-seq and microarray data are available under GEO accession number GSE60356.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, five tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.02.003.

AUTHOR CONTRIBUTIONS

C.C.B. designed the studies, performed most of the experiments, analyzed the data, and wrote the manuscript. D.E., M.L., and D.M. performed and analyzed gut-inflammation studies. A.S., I.O.-G., and R.a.-B. assisted in processing of samples for in vitro co-culture studies and qPCR. R.E. and C.O. assisted with processing of tissues for phenotyping studies. M.A. provided advice and performed microarrays. V.P. analyzed ChIP sequencing data. E.d.R. supervised ChIP-seq data analysis. G.M.L. provided advice and supervision. D.M. designed gut-inflammation studies and contributed to the writing of the manuscript. R.J.N. supervised the overall study and contributed to the writing of the manuscript.

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Immunity

Supplemental Information

Retinoic Acid Is Essential for Th1 Cell Lineage Stability and Prevents Transition to a Th17 Cell Program

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Figure S1 (related to Figure 1). Expression of Foxp3 in $CD4^+$ T-cells deficient in RA signaling

(A) Intracellular expression of Foxp3 in $CD4^+$ T-cells from spleen, thymus and mesenteric lymph nodes (MLN) of wild-type littermate control (WT) and dn*Rara* mice.

(B) Total number of $CD4^+Foxp3^+$ T-cells in spleen (upper panel) and thymus (lower panel) of WT and dn*Rara* mice

Data are representative of two independent experiments. Mean ± SEM.

Figure S1.

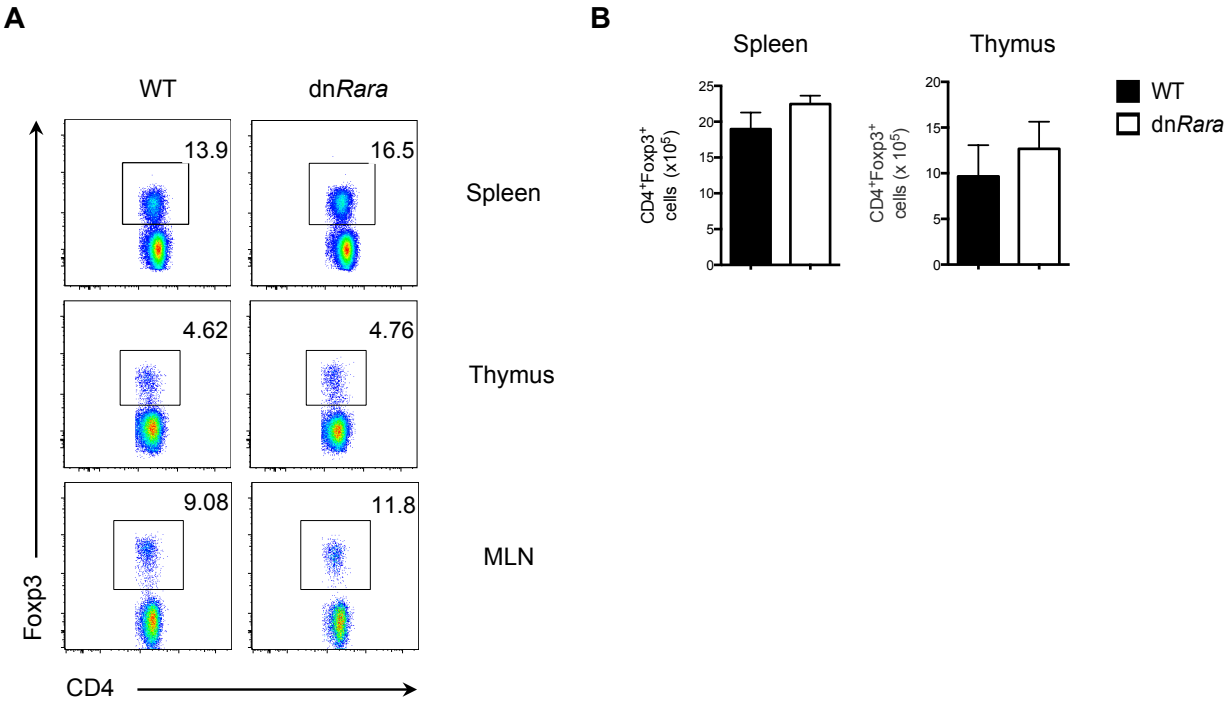

A
WT
dnRara
13.9
16.5
Spleen
4.62
4.76
Thymus
9.08
11.8
MLN
Foxp3
CD4
B
Spleen
Thymus
CD4+Foxp3+ cells (x10^5)
CD4+Foxp3+ cells (x 10^5)
WT
dnRara

Figure S2 (related to Figure 2). Proliferation and differentiation of CD4$^+$ T-cells in the absence of RA signaling

(A) Naïve CD4+ T-cells from WT and dn*Rara* mice were labeled with CellTrace^{TM} and cultured under Th1 conditions for 5 days. Flow cytometry showing dye dilution, gated on viable CD4$^+$ T-cells.

(B) Cell-surface expression of CD44 and CD25 on naïve CD4+ T-cells from WT or dn*Rara* mice cultured under Th1 conditions for 5 days.

(C) Naïve CD4+ T-cell from WT and dn*Rara* mice were cultured under Th0 or Th2 conditions for 6 days. Cells were analysed by flow cytometry for expression of intracellular RORγt. Gated on CD4$^+$ T-cells.

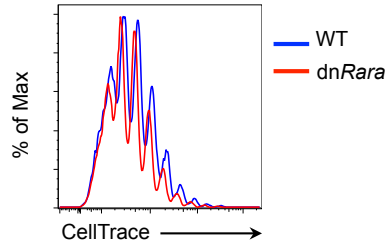
(D) Sorted naïve CD4$^+$ T- cells from WT and dn*Rara* mice were cultured under Th17 conditions for 6 days. Intracellular IL-17A and IFN-γ expression after stimulation with PMA and ionomycin.

(E) CD4$^+$ T-cells from dn*Rara*-*Ifng*^{eYFP} and *Ifng*^{eYFP} mice were cultured under Th1 conditions. Quantitative real-time PCR analysis of *Cxcr3* and *Il12rb2* from IFN-γ$^+$ (eYFP$^+$) cells sorted on day 7. Samples from three independent experiments.

Representative data from two to three independent experiments (A-D). Mean ± SEM.

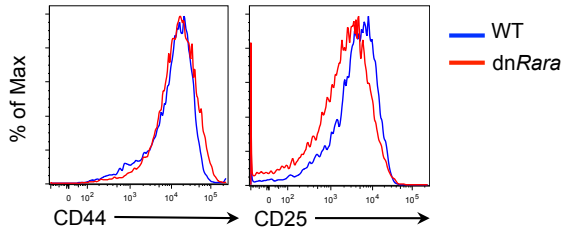
Figure S2.

A

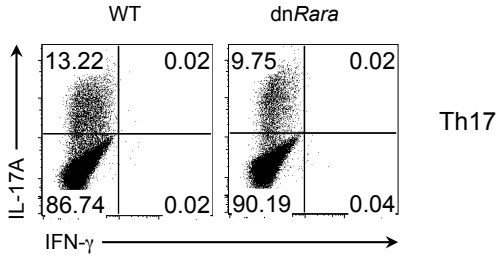


	WT	dn*Rara*
% cells divided	90.83	92.67
Proliferation index	3.65	3.86

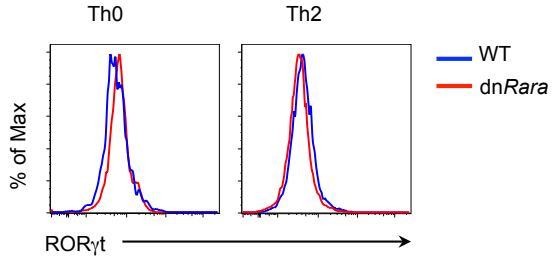
B



C



D



E

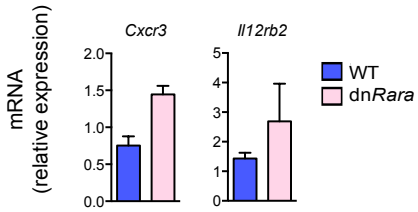


Figure S3 (related to Figure 3). STAT3 and STAT4 activity in dn*Rara* Th1 differentiated cells

(A) Flow cytometric analysis of STAT3 and STAT4 phosphorylation in naïve $CD4^+$ T-cells from dn*Rara* and WT mice differentiated under Th1 conditions. Cells analysed after 6 days following treatment with 25ng/ml IL-12, 20ng/ml IL-6 and 10ng/ml IL-23 for 30 minutes. Dashed lines represent untreated cells.

(B) Bar graph depicts ratio of pSTAT3/pSTAT4 signaling as assessed by MFI.

Figure S3.

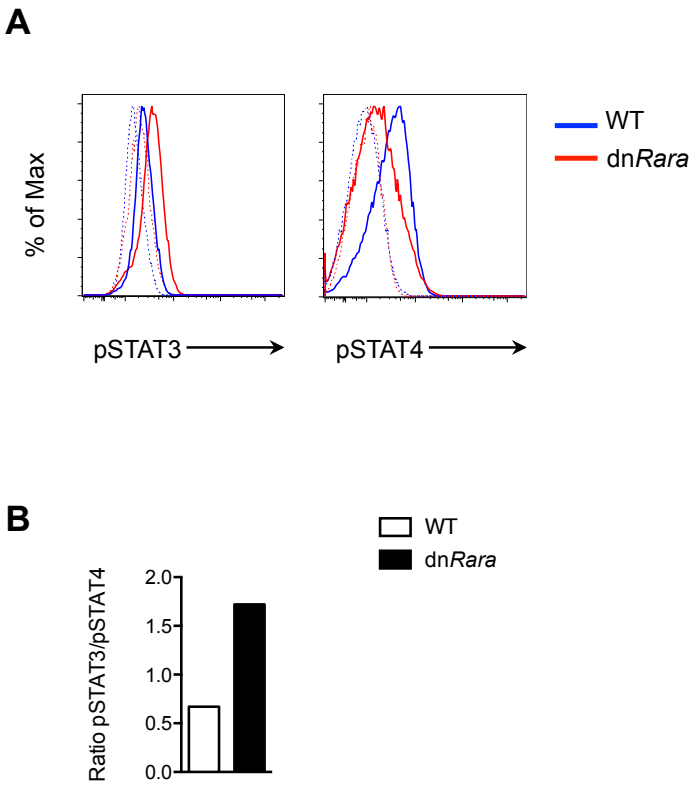

A
% of Max
pSTAT3
pSTAT4
WT
dnRara
B
WT
dnRara
Ratio pSTAT3/pSTAT4
2.0
1.5
1.0
0.5
0.0

Figure S4 (related to Figure 4). Cytokine analysis following temporal inhibition of RA signaling in Th1 cells

(A) Naive $CD4^+$ T-cells from dn*Rara*$^{lsl/lsl}$ mice were cultured under Th1 conditions. Th1 cells were transduced with TAT-Cre on days 5 and 7 and repolarised under Th1 conditions for a further 5 days. Intracellular expression of IFN-γ and IL-17A following PMA and ionomycin stimulation.

(B) Naive CD4+ T-cells from *Ifng*^{eYFP} mice were differentiated under Th1 conditions. IFN-γ^+ ($eYFP^+$) cells were sorted on day 7 and recovered cells underwent secondary repolarisation in Th1 conditions for 5 days in the presence of Veh or RAi. Intracellular expression of IFN-γ and IL-17A following PMA and ionomycin stimulation.

Data representative of two independent experiments.

Figure S4.

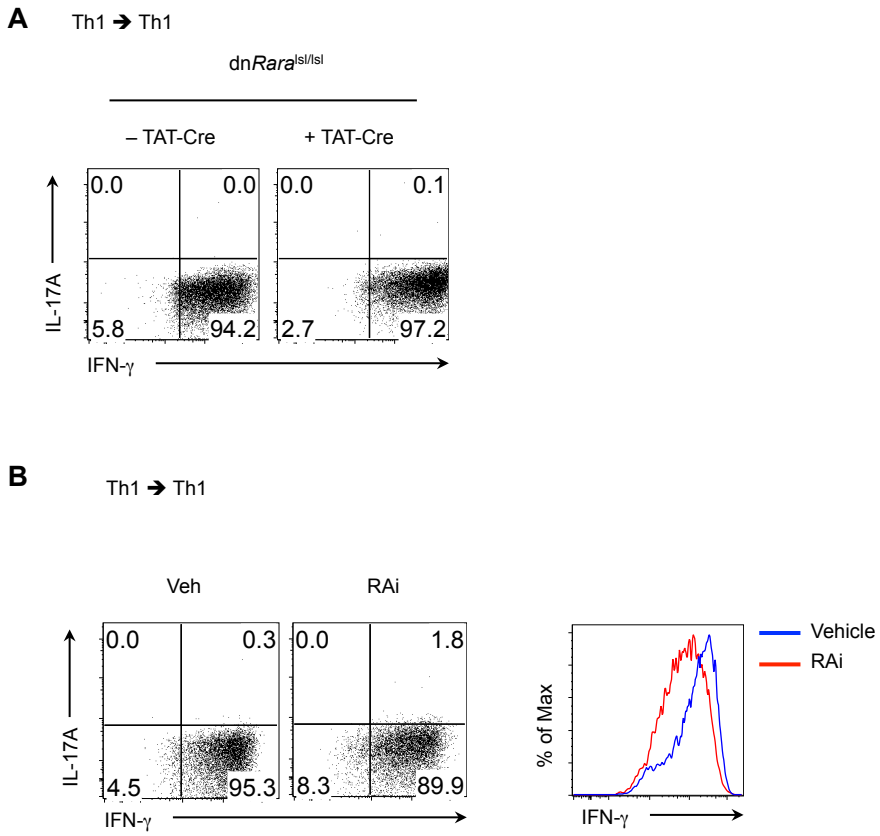

A
Th1 ➔ Th1
dnRara lsl/lsl
– TAT-Cre
+ TAT-Cre
0.0
0.0
0.0
0.1
5.8
94.2
2.7
97.2
IL-17A
IFN-γ
B
Th1 ➔ Th1
Veh
RAi
0.0
0.3
0.0
1.8
4.5
95.3
8.3
89.9
IL-17A
IFN-γ
% of Max
IFN-γ
Vehicle
RAi

Figure S5 (related to Figure 5). RA-RARα regulates enhancers at Th1 genes and represses Th17 lineage specifying genes

Naive $CD4^+$ T-cells from dn*Rara* and WT mice were cultured under Th1 conditions as in Figure 5. After 6 days, ChIP was performed with the specified antibodies, followed by real-time PCR analysis at selected sites (B-C) or sequencing (A).

(A) ChIP-seq binding tracks at *Stat4* and *Ifng* loci for RARα in WT Th1 polarised cells and p300 binding, H3K27ac, H3K4me1 and H3K4me3 modifications in WT and dn*Rara* Th1 cells.

(B) Validation of the RARα ChIP-seq regions in (A) by ChIP-qPCR assays. Untr6 region serves as a negative control. Data presented normalised to input.

(C) ChIP analysis of the abundance of p300 at the loci in (B) in WT and dn*Rara* Th1 cells. Data presented normalised to input.

(D) ChIP-seq analysis of STAT4 binding at the Tbx21 enhancer and comparison of p300 binding in WT and $STAT4^{-/-}$ Th1 cells. ChIP-Seq data (Vahedi et al. 2012 and Wei et al., 2010) was mapped to the Dec. 2011 (GRCm38/mm10) mouse genome assembly with the UCSC genome browser along with the ChIP-seq binding track for RARα at the *Tbx21* locus.

(E) Quantitative real time PCR analysis of selected genes identified as differentially expressed on genome wide transcriptional profiling analysis of cells as in (A). Mean ± SEM.

(F) Cell-surface expression of IL6-Rα by flow cytometry in naïve dn*Rara* and WT $CD4^+$ T-cells at indicated timepoints. Grey histogram indicates staining for isotype control.

Data (B-F) representative of two to three independent experiments. Mean ± SD unless otherwise stated, $^{**}p < 0.01$; $^{****}p < 0.0001$.

Figure S5.

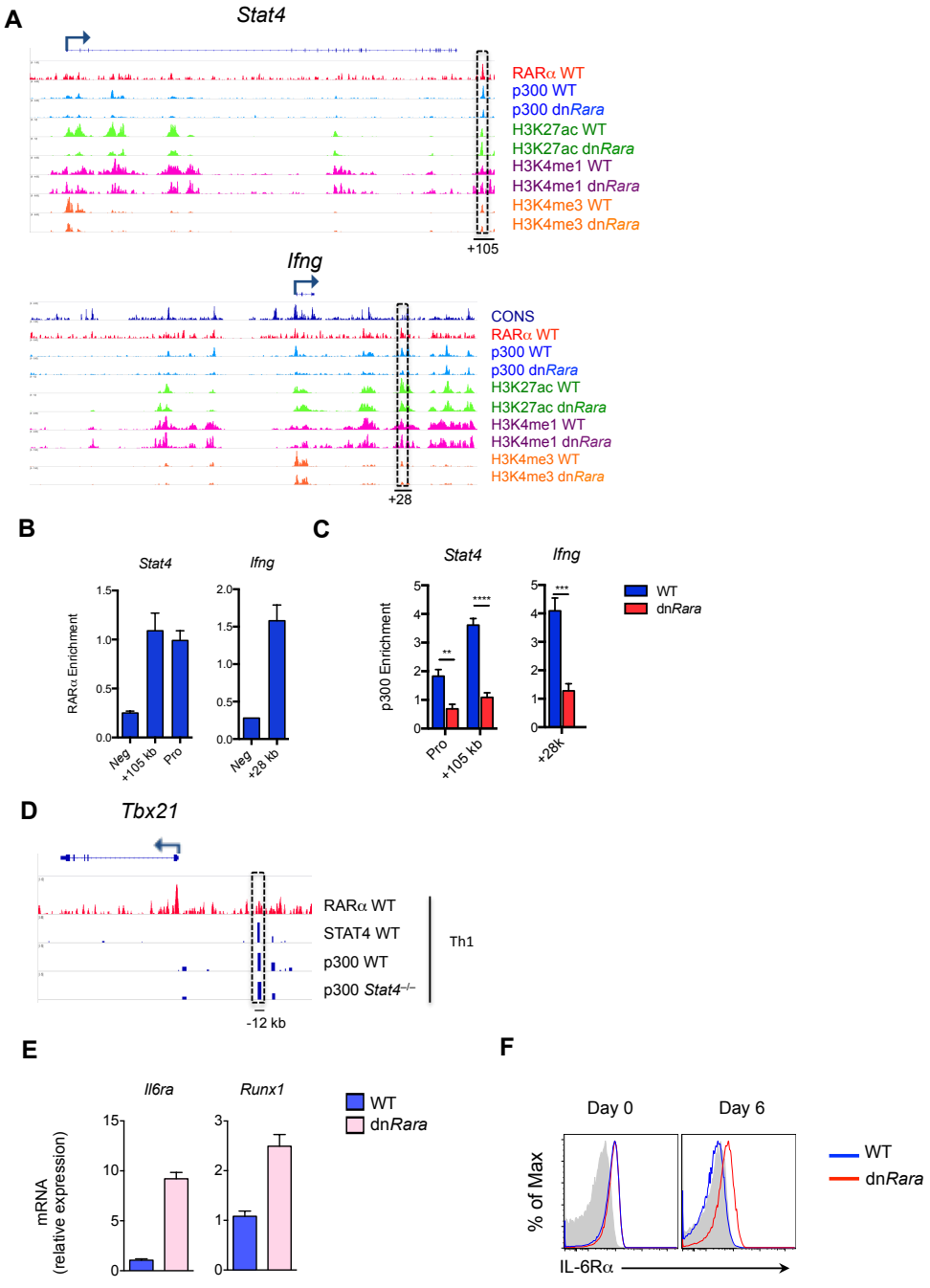

A
Stat4
RARα WT
p300 WT
p300 dnRara
H3K27ac WT
H3K27ac dnRara
H3K4me1 WT
H3K4me1 dnRara
H3K4me3 WT
H3K4me3 dnRara
+105
Ifng
CONS
RARα WT
p300 WT
p300 dnRara
H3K27ac WT
H3K27ac dnRara
H3K4me1 WT
H3K4me1 dnRara
H3K4me3 WT
H3K4me3 dnRara
+28
B
Stat4
Ifng
RARα Enrichment
Neg
+105 kb
Pro
Neg
+28 kb
C
Stat4
Ifng
p300 Enrichment
Pro
+105 kb
+28k
WT
dnRara
D
Tbx21
RARα WT
STAT4 WT
p300 WT
p300 Stat4−/−
Th1
-12 kb
E
Il6ra
Runx1
mRNA (relative expression)
WT
dnRara
F
Day 0
Day 6
% of Max
IL-6Rα
WT
dnRara

Figure S6 (related to Figure 6). Cytokine production by dnRARa T-cells following infection with L. monocytogenes

(A) Splenocytes from dn*Rara* and WT mice infected with Lm-2W were restimulated with LLOp for 24 h. Concentration of IFN-γ, IL-17A and IL-4 in supernatants was measured by multiplex bead array (Biorad). Data normalised to total numbers of $CD4^+$ T-cells. n = 3-4 mice per group.

(B) Intracellular staining for IFN-γ and IL-4 following stimulation of splenocytes with LLOp for 6 h, 7 days after infection with *L. monocytogenes*. Gated on $CD3^+CD4^+$ T-cells

(C) Cell surface expression of IL-6Rα by flow cytometry on LLOp:I-A^b CD4+ T-cells isolated from spleen of dn*Rara* or WT mice 7 days after infection with *L. monocytogenes*. Data from 4 pooled mice. Numbers indicate MFI.

Data representative of two to three independent experiments. Mean ± SEM.

Figure S6.

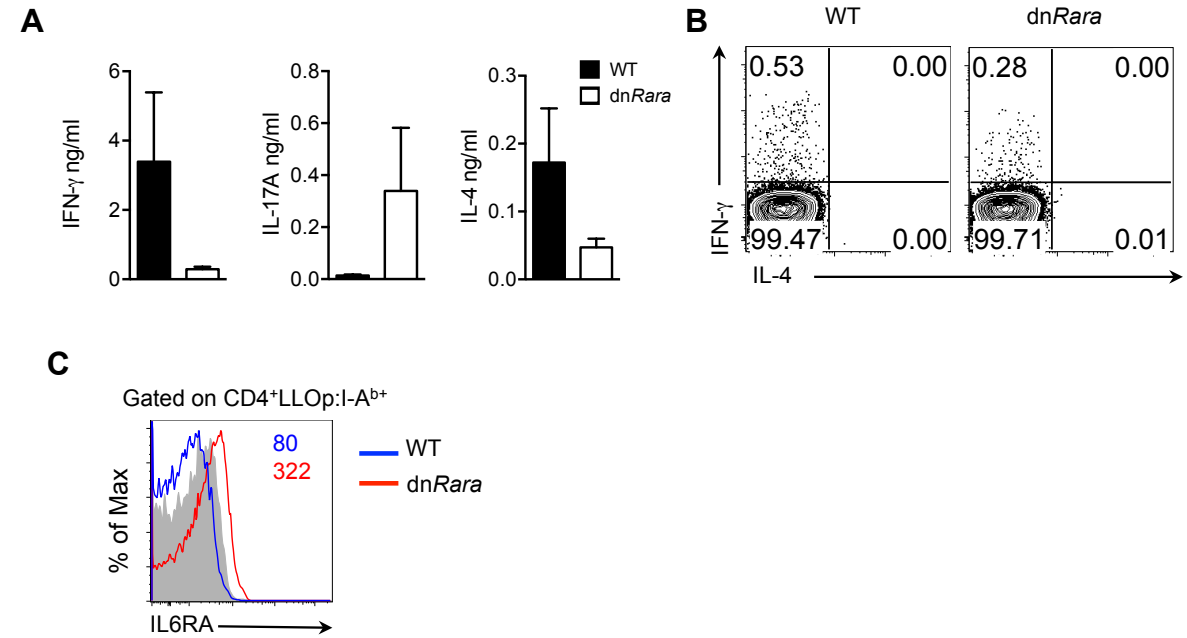

A
IFN-γ ng/ml
IL-17A ng/ml
IL-4 ng/ml
WT
dnRara
B
WT
dnRara
0.53
0.00
99.47
0.00
0.28
0.00
99.71
0.01
IFN-γ
IL-4
C
Gated on CD4+LLOp:I-Ab+
% of Max
80
322
WT
dnRara
IL6RA

Figure S7 (related to Figure 7). Gut homing in dn*Rara*-OTII CD4+ T-cells
(A) Percentage of OTII or OTII(dn*Rara*) $CD4^+$ cells recovered from LPL, IEL, MLN and Spleen of $RAG^{-/-}$ recipients, 9 days after adoptive transfer (n = 3-4 per group). Data representative of two independent experiments. Mean ± SEM.

Figure S7.

A

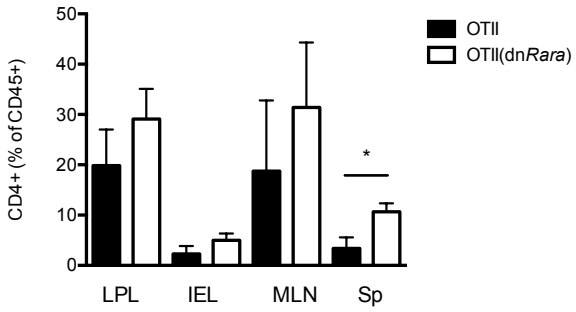


Table S1 (related to Figure 5). List of Sequencing-Based Data Used in This Study including publically available data as indicated by Geo Accession Number

Samples	Non-redundant tags	Peak counts
RARA_WT	13303876	1776
H3K4me1_DNRAR	18605274	65960
H3K4me1_WT	23760603	49542
H3K4me3_DNRAR	18333386	49505
H3K4me3_WT	21918629	53135
H3K27Ac_DNRAR	17421600	37788
H3K27Ac_WT	20513640	37151
H3K27me3_DNRAR	30667883	56002
H3K27me3_WT	20833021	78511
p300_DNRAR	23023765	30495
p300_WT	25213927	46191
Stat4 WTTh1 (GSM550303)	8982352	20862
p300 WT Th1 (GSM994508)	19652779	25554
p300 Stat4$^{-/-}$ Th1 (GSM994509)	18282554	29208

Table S2 (related to Figure 5). Genes downregulated in dn*Rara* Th1 cells that were bound by RARα in WT Th1 cells.

1110037F02Rik	Fli1	Ncln
1810011H11Rik	Fmnl3	Nedd4l
3300005D01Rik	Foxo3	Nfic
5830416P10Rik	Foxp1	Nln
Acsl4	Furin	Nme1
Adora2a	Gas5	Nod1
Alkbh7	Gcsh	Notch2
Asb2	Gfi1	Nt5e
Birc5	Gimap3	P2rx7
Blm	Gimap4	Pde2a
Bre	Gimap8	Prr5l
Capzb	Gimap9	Rbks
Chsy1	Hic1	Rcbtb2
Cmas	Hmgcs1	Shf
Cnga1	Idi1	Slc16a6
Coq7	Ifngr1	Smad3
Ctps	Ifrd2	Sqle
Cycs	Irf8	Sulf2

Cyp51	Itih5	Tbx21
Cyp51	Kcnn4	Treml2
Dennd4a	Kif2c	Txn2
Dusp6	Lbr	Ube2e3
E2f3	Lef1	Uchl3
Enpp4	Mdc1	Vav3
Fasn	Me2	Vipr1
Fgl2	Mrto4	

Table S3 (related to Figure 5). Genes upregulated in dn*Rara* Th1 cells that were bound by RARα in WT Th1 cells

1110038F14Rik	Ifngr2	Slfn2
Ak2	Il15ra	Socs1
Antxr2	Insr	Sp100
Aph1b	Irf1	Stat1
Arhgap25	Irgm1	Tagap
Arid4a	Kif3b	Tmem50a
B2m	Mcl1	Tnip1
Bace2	Mettl8	Tor1aip2
Bcl10	Mga	Traf1
Bcl6	Mpeg1	Trpm6
Birc3	Nek6	Twsg1
Cd320	Net1	Usp53
Cnnm2	Npc2	Vav1
Ddit3	Plec	Wdsub1
Egr2	Polg	Zbp1
Fam43a	Ptpn1	Zfp207
Filip1l	Rab19	Zfp36l2
Fndc3a	Rhd	Zmym6
Fuca1	Slamf1	

Table S4. Taqman assays used for RT-PCR gene expression analyses (related to Figures 1-3 and 5).

Mouse ACTB	4352341E
Il6ra	Mm00439653_m1
Il22	Mm00444241_m1
Runx1	Mm01213404_m1
Batf	Mm00479410_m1
Cxcr3	Mm99999054_s1

Il23r	Mm00519943_m1
Il1r1	Mm00434237_m1
Il21	Mm00517640_m1
Il10	Mm00439616_m1
Irf8	Mm00492567_m1
Irf4	Mm00516431_m1
Stat4	Mm00448890_m1
Il12rb2	Mm00434200_m1
Ifng	Mm00801778_m1
Il12rb1	Mm00434189_m1
Rorc	Mm01261022_m1
Gata3	Mm00484683_m1
Tbx21	Mm00450960_m1

Table S5 (related to Figure 5). Sequences of PCR primers used in ChIP assays

Stat4_+105k F	TCCTCCTCCCTTTGTTGTTC
Stat4 +105k R	GGGCCTTAATCAACCATTTC
Stat4 Promoter F	AGAGGGCATACACCGAGAAC
Stat4 Promoter R	TCTAGGGAGCCAGCATCAAC
Tbx21 Promoter F	TCGCTTTTGGTGAGGACTG
Tbx21 Promoter R	GGTGGCAGGTTGACTCTTTC
Tbx21 -12k F	GCGGAAGAGGGAACTAACAC
Tbx21 -12k R	GGACCCGGAACCTATGTATG
Irf8 Promoter F	CAGAAGCTAGGGCTGGTGTC
Irf8 Promoter R	CACAGAACAGATCCCAAATGTC
Irf8 -11k F	CCTTAACCCCGGAACTGTAG
Irf8 -11k R	TGCTGTGCTTGCCTCTACTC
Il6ra Promoter F	TCCGCTTGAGTTTTGCTTTC
Il6ra Promoter R	CACTGACCTGCCTTCTACTTTAAC
Il6ra +32k F	CAAAGCTAAAACCAGGAAATGAC
Il6ra +32k R	AAAAGGTTCCATGTGATGTTG
Rorc Promoter (Rorγt isoform) F	AGGAATTTGGGTGTGGTGAG
Rorc Promoter (Rorγt isoform) R	CTGTCTTGGGTGGTGTCTTG
Runx1 Promoter 1 F	TGGAAGAGGAAGAAGCTGTG
Runx1 Promoter 1 R	CAAGAGAAGCCACCCCAAAC
Runx1 Promoter 2 F	TGCTGGGCTTACACTTCTGAC
Runx1 Promoter 2 R	TGGACCTCATAAACAACCACAG
IFNg +28k F	CTTTGAGCCACTGATGGGTAG
IFNg +28k R	GCCTCTCCACGTCTCTTCTTC

Supplemental Experimental Procedures

Reagents

$LLO_{190-201}$ was synthesised by PiProteomics and was >95% pure, as determined by HPLC. LLO:I-A^b monomers were provided by NIH Core Tetramer Facility. PE labeled LLO:I-A^b dextramers were synthesised by Immudex. Recombinant Lm-2W strain was provided by Marc Jenkin's Laboratory. LE540 was purchased from Alpha Laboratories.

Naïve CD4+ T-cell isolation and culture

Naïve $CD4^+CD25^{neg}CD44^{lo}CD62L^{hi}$ T-cells were isolated by cell sorting by FACSAria (BD) after enrichment with a $CD4^+$ T-cell negative selection kit (Miltenyi Biotec). T-cell depleted splenocytes were prepared using a $CD3^+$ microbead selection kit (Miltenyi Biotec) followed by irradiation at 3000 rad. Naïve CD4+ T-cells were cultured for 3 days with irradiated T cell-depleted splenocytes at a ratio of 1:5 in the presence of 5 µg/ml of anti-CD3 (145-2C11) under Th0 cell conditions (IL-2 100 IU/ml, anti-IL-4 (11B11) and anti-IFN-g (XMG1.2), 10 µg/ml each); Th1 cell conditions (100 IU/ml of IL-2, 10 ng/ml of IL-12, and anti-IL-4); Th2 cell conditions (100 IU/ml of IL-2, 10 ng/ml of IL-4, anti-IL-12 (C17.8), and anti-IFN-γ (XMG1.2); or Th17 cell conditions, 5 ng/ml TGFβ, 20 ng/ml IL-6, 10 ng/ml IL-1β, anti-IL-4, and anti-IFN-γ). Cells were expanded for an additional 3-4 days. Where indicated, 10 ng/ml IFN-γ or 10 µg/ml anti-IFN-γ was added. In secondary repolarisation assays, where specified, LE540 (1 µM) or DMSO (vehicle control) was added to the media. Cytokines were from R&D. Anti-CD3 was from BioXcell and other antibodies were from BD Biosciences. All cell cultures were performed in complete RPMI containing 10% fetal

bovine serum (FBS), 55 μM β-mercaptoethanol, HEPES, non-essential amino acids, glutamine, penicillin and streptomycin.

TAT-Cre transduction

Sort purified naïve CD4+ T-cells were differentiated under Th1 conditions. After 5 days, cells were washed twice in serum free medium prior to treatment with 50 μg/ml TAT-Cre (Millipore) or medium alone (mock treatment). Cells were incubated at 37°C for 45 minutes. The reaction was quenched with medium containing 20% FBS followed by further washing. Cells were expanded for 2 days followed by retreatment with TAT-Cre or media as before. Cells were then restimulated under Th1 cell conditions for 3 days and expanded for a further 2 days prior to analysis.

Flow Cytometry

For analysis of cytokine production, cells were restimulated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin in the presence of monensin for 4-5 h at 37^{o}C in a tissue culture incubator. Cell surface staining was carried out in PBS with 2% FBS. For live cell analysis or cell sorting, dead cells were excluded by staining with SYTOX blue (Invitrogen). For intracellular staining, cells were first stained with LIVE/DEAD Fixable Violet or near IR Dead Cell Stain (Invitrogen), followed by staining for cell-surface markers and then resuspended in fixation/permeabilisation solution (Cytofix/Cytoperm kit or Transcription Factor Buffer kit; BD Bioscences). Intracellular staining carried out in accordance with the manufacturer's instructions. Intracellular phosphorylated STAT proteins were stained with Phosflow Lyse/Fix Buffer, and Phosflow Perm Buffer III (BD

Biosciences) according to the manufacturer's protocol. Data were collected with a LSR Fortessa (BD) and results were analyzed with FlowJo software (Tree Star). All the antibodies for staining cell surface markers, cytokines or transcription factors were purchased from either BD Biosciences or eBiosciences.

Luminex Immunoassays

Cytokine levels in supernatants were measured using a multiplex bead-based assay (Bio-Rad Laboratories) in a Luminex FlexMap3D System (Luminex Corporation).

Western Blotting

Differentiated Th1 cells were lysed in RIPA buffer supplemented with protease inhibitors. Lysates were electrophoresed on 10% gels (Biorad), transferred to nitrocellulose and blotted with anti-STAT4 or anti-actin followed by anti-rabbit-horseradish peroxidase conjugated antibody. All antibodies were from Cell Signaling Technology.

***L. monocytogenes* infection and analysis**

Mice were infected i.v. with 1 x 10^6 cfu L. monocytogenes and spleens were harvested 7 days later. For FACS analysis, single cell suspensions were enriched for $CD4^+$ T-cells with a $CD4^+$ T-cell negative selection microbead kit (Miltenyi Biotec) and stained with PE labeled, LLO:I-A^b dextramer (Immudex) and cell surface antibodies. For analysis of cytokine production, supernatants were collected from splenocytes restimulated with LLO peptide (PiProteomics) at 10 µg/ml for 24 h or intracellular cytokine staining was performed following stimulation with LLO peptide for 6 h in the presence of monensin.

Food antigen induced diarrhoea model

Rag1$^{-/-}$ mice were kept on a sulfatrim-containing diet and only exposed to autoclaved supplies. Naïve OTII CD4 cells (defined as $CD4^{+}CD25^{-}Vb5^{+}Va2^{+}CD44^{-}$) were sorted from 8-12 weeks old female C57Bl6 OTII(dn*Rara*) or C57Bl6 OTII mice using a FACS Aria cell sorter (Becton Dickinson), and 2 x 10^6 cells in 100µl PBS were retro-orbitally transferred to 12 weeks old Rag1$^{-/-}$ females. 12h after the adoptive transfer, the drinking water was replaced by a 1% Grade II ovalbumin (OVA, Sigma) and 0.5% Splenda (McNeil Nutritionals) solution for 7 days. Body weight was measured at 5pm every day. For monitoring diarrhea development, the faeces texture after 7 days of OVA, 2h after a gavage challenge with 50mg Grade III OVA (Sigma) in 200 µl PBS on days 9 and 10 and without further challenge on day 12 was analysed. A mouse was diagnosed with diarrhoea if the faeces had the characteristic soft and light appearance at two consecutive occasions. For the single gavage challenge experiment, mice were subjected to the challenge on day 9 only and the faeces were analysed after 2h. To determine T cell frequencies, lymphocytes were isolated as previously described (Mucida et al., 2007) on day 7 (from mesenteric lymph node (MLN) and spleen only) or day 9 (from the intestinal epithelium, lamina propria, MLN and spleen) after the start of oral OVA exposure of the recipient mice. For cytokine staining, isolated lymphocytes were stimulated for 3h in RPMI medium supplemented with 10% FBS, 55 µM β-mercaptoethanol, 100ng/ml PMA (Sigma), 500ng/ml Ionomycin (Sigma) and 10µg/ml brefeldin A (Sigma) prior to the incubation with antibodies. Cells were first stained with antibodies against cell surface markers, followed by permeabilization using either Fix/Perm buffer (BD Pharmingen) for cytokine stainings, or

using the Foxp3 Mouse Regulatory T cell Staining Kit (eBioscience) for Foxp3 staining. The fluorescent-dye- conjugated antibodies used were obtained from BD-Pharmingen (anti-CD4, 550954; anti-CD25, 553866; anti-IL-17a, 559502; anti-Vb5, 553190) or eBioscience (anti-CD44, 56-0441; anti-CD45.2, 47-0454; anti-TCR-β, 47-5961; anti-IFN-γ, 25-7311; anti-Foxp3, 17-5773; anti-Vα2, 48-5812). Stained cells were analysed using a LSR-II flow cytometer (Becton Dickinson) and population frequencies were determined using the FlowJo software (Tree Star).

Chromatin immunoprecipitation (ChIP)

20-60 million Th1 polarised cells from WT and dnRara mice were fixed, washed and snap-frozen according to the Cell Fixation protocol from Active Motif (http://www.activemotif.com/documents/1848.pdf). Chromatin was isolated by the addition of lysis buffer, followed by disruption with a Dounce homogenizer. Lysates were sonicated and the DNA sheared to an average length of 300-500 bp. Genomic DNA (Input) was prepared by treating aliquots of chromatin with RNase, proteinase K and heat for de-crosslinking, followed by ethanol precipitation. Pellets were resuspended and the resulting DNA was quantified on a NanoDrop spectrophotometer. Extrapolation to the original chromatin volume allowed quantitation of the total chromatin yield. An aliquot of chromatin was precleared with protein A agarose beads (Invitrogen). Following immunoprecipitation with specified antibodies, complexes were washed, eluted from the beads with SDS buffer, and subjected to RNase and proteinase K treatment. Crosslinks were reversed by incubation overnight at 65 C, and ChIP DNA was purified by phenol-chloroform extraction

and ethanol precipitation and used for the preparation of Illumina sequencing libraries and for ChIP qPCR analysis.

ChIP-qPCR

Quantitative PCR (qPCR) reactions were carried out in triplicate on specific genomic regions using SYBR Green Supermix (Bio-Rad). See Table S5 for Primer details. The resulting signals were normalized for primer efficiency by carrying out qPCR for each primer pair using Input DNA. By using standards of known quantities of DNA it was possible to calculate the number of genome copies pulled down for each of the sites tested, and thus to calculate the copies pulled down per starting cell number, presented as 'Enrichment'. For RARα ChIP qPCR a gene desert on chromosome 6 (Untr6) was used for a negative control site (Active Motif Catalog No: 71011).

ChIP Sequencing (Illumina)

Illumina sequencing libraries were prepared from the ChIP and Input DNAs using standard procedures and libraries were sequenced on HiSeq 2500

ChipSeq Analysis

For each sample the 50bp SE reads in FastQ format from the sequencer were aligned to the mouse reference genome (mm10) using Novoalign v2.07.11 (http://www.novocraft.com). The resulting alignment file was converted to BAM format using samtools (http://samtools.sourceforge.net/) and the pcr duplicates were removed using picard tools (http://picard.sourceforge.net). Only uniquely mapped reads from each

sample were selected for further analysis. Significantly enriched regions from each sample were identified with MACS v2.0.10_20131216 (Zhang et al. 2008, Feng J et al. 2011) (with q=0.10) using the input sample for background correction. In some instances peaks were identified by visual inspection and confirmed by ChIP qPCR. In case of H3K4me1 and H3K27me3 samples, "--broad" setting was used to merge nearby enriched regions. For visualization purposes, the input signal was subtracted from each ChIP sample and was converted into bigWig format using "bedGraphToBigWig" utility from UCSC tools (http://genome.ucsc.edu/util.html). The identified significantly enriched regions were annotated to find the associated genes using "FindNeighbouringGenes" utility from USeq package (http://useq.sourceforge.net/). Associated genes represent the closest transcriptional start site from the centre of the peak.

Microarray data

Total RNA was extracted from cells lysed in Trizol LS reagent (Life Technologies). RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies) and quantified with the Nanodrop ND-1000 UV-spectrophotometer (NanoDrop Technologies).

Transcriptome in IFN-γ^+ (eYFP$^+$) CD4$^+$ T-cells

Naïve CD4$^+$ T-cells from dn*Rara*-IFN-γ^{eYFP} or littermate control IFN-γ^{eYFP} reporter mice were cultured under Th1 conditions. On day 7 of culture, following restimulation with PMA and ionomycin, eYFP$^+$ cells were sorted and total RNA was extracted for transcriptional profiling using Affymetrix Mouse Gene 2.0 ST arrays. Pre-processing and statistical analysis of gene expression data were done using Partek Genomics Suite 6.6. CEL files

were imported and expression intensities were summarised, normalised and transformed using Robust Multiarray Average algorithm. Two additional samples from eYFP$^+$ dn*Rara* or wild-type cells sorted without prior restimulation were included in the normalisation. These samples were not included in the analysis of differentially expressed genes. P values <0.05 and fold change in expression ≥ 1.5 or ≤ -1.5 were considered significant.

Transcriptome in Th1 differentiated cells

Sorted naïve CD4$^+$ T-cells from dn*Rara* or WT mice were polarised under Th1 conditions. On day 6 of culture cells were harvested and total RNA was extracted for microarray study or ChIP. RNA isolation, microarray and data processing performed by Miltenyi Biotec. Transcriptome analysis was performed using Agilent Whole Mouse Genome Oligo Microarrays 8X60K in accordance with manufacturer's protocol. Data analysis was performed using R/bioconductor and software packages therein (http://www.R-project.org ; http://www.bioconductor.org) or MS-Office Excel (Microsoft Inc.). Background corrected intensity values were normalized between arrays using quantile normalization. Quality controls include comparison of intensity profiles and a global correlation analysis. Differentially expressed genes were identified by statistical group comparisons on normalized (background corrected and quantile normalized) log2 transformed fluorescence intensities using Student´s t-test (two-tailed, equal variance). Reporters showing a p-value ≤ 0.05 and a median fold-change in expression ≥ 1.5 or ≤ -1.5 were considered as reliable candidates for altered gene expression. In addition, at least two of the replicate samples in the group with higher expression were required to have detection p-values ≤ 0.01.

Supplemental References

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Mucida, D., Park, Y., Kim, G., Turovskaya, O., Scott, I., Kronenberg, M., Cheroutre, H. (2007) Reciprocal TH17 and Regulatory T Cell Differentiation Mediated by Retinoic Acid. Science *317*, 256-260

Yong Zhang, Tao Liu1, Clifford A Meyer, Jérôme Eeckhoute, David S Johnson, Bradley E Bernstein, Chad Nusbaum, Richard M Myers, Myles Brown, Wei Li7 and X Shirley Liu. (2008) Model-based Analysis of ChIP-Seq (MACS). Genome Biology. 9:R 137

CHAPTER 3: RARα orchestrates activation of lineage specific enhancers to regulate T helper cell fate

Abstract

Enhancers are critical for directing cell-type-specific transcriptional programs. Differentiation of naïve $CD4^+$ T cells into lineages requires the co-ordinated actions of transcription factors and epigenetic modifiers to impart lineage stability through stable changes in gene expression. Our findings in Chapter 2 indicate that RA/RARα can regulate gene expression through the direct modulation of enhancer elements at key Th1 and Th17 genes. In Chapter 3, we perform a genome wide analysis of RARα regulation of T helper cell enhancers, mapping RARα and enhancer activity in $CD4^+$ T cells at different stages of differentiation. The data establish that RARα plays a critical role in the activation of lineage-specific enhancers. During Th1 and Th17 differentiation, RARα is recruited to cell-specific enhancers where it acts, in part, by recruiting the coactivator, p300. Thus, RARα plays a broad role in the regulation of T helper cell fate and function, providing a molecular basis for the pleiotropic and seemingly paradoxical effects of RA on T helper cell fate.

Background

The data presented in Chapter 2 identified a critical role for RARα signaling in the maintenance of Th1 cell fate, and identified RARα regulation of key Th1 genes with reciprocal regulation of the Th17 program. At selected genes, RARα was shown to regulate transcription through activation of enhancer regions. These findings suggest that enhancer regulation may be the key mechanism by which RARα influences gene expression and cell fate. A recent study identified a role for STAT proteins in the regulation of T helper cell enhancers (Vahedi et al., 2012). However, it is not clear whether STATs bind p300 directly, and the mechanisms by which coactivators are recruited to specific enhancer sites remain incompletely understood. Shortly after publication of Chapter 2, a study of oestrogen-receptor-regulated enhancers demonstrated that RARα formed part of a complex of transcription factors which are recruited to, and help to regulate, oestrogen-receptor dependent enhancers (Liu et al., 2014). A number of the coactivators recruited by the protein complex were dependent on RARα. These findings hint at a broader role for RARα in the regulation of lineage specific enhancers, through recruitment of cofactors which mediate epigenetic modifications.

The observed repression of a Th17 program in Th1 cells is consistent with the previously reported reciprocal regulation of iTreg and Th17 differentiation by RARα. However, VAD mice have a near absence of Th17 cells, suggesting a requirement for RA in Th17 cell differentiation. Therefore, the physiological importance of RA/RARα during Th17 differentiation is

unresolved. The studies in this chapter start to address cell-context dependent actions of RARα on Th17 genes.

Here we report a comprehensive analysis of genomic targeting by RARα in T helper cell subsets. Our findings identify RARα as a pervasive regulator of enhancer regions with p300 recruitment critically dependent on RA signaling. Furthermore, RARα is invariably present at super-enhancer regions where its presence is required for activation of these enhancers. Remarkably, integration of our data with previously published ChIP-seq studies identifies RARα as the dominant regulator of enhancer activity. Genome-wide mapping of RARα binding and enhancers in T helper cell subsets reveals that RARα targets cell-type-specific enhancers in Th1 and Th17 cells, providing a mechanistic basis for the context dependent effects of RA/RARα on Th17 differentiation. RARα is a tantalizing pharmacological target in the treatment of immune mediated diseases. A number of selective RARα agonists and antagonists are in development. Our findings shed light on the mechanisms by which RARα regulates transcriptional pathways and will guide the rational use of RARα modulators in clinical practice.

RESULTS

RARα opposes alternative cell fates in Th1 cells

In previous chromatin immunoprecipitation followed by sequencing (ChIP-Seq) studies (**Chapter 2**) we identified 1776 RARα binding sites in Th1 cells. However, ChIP-seq studies for RARs in other cell types suggest that these nuclear receptors are usually bound at several thousand sites across the genome (Liu et al., 2014). We therefore performed further ChIP-seq experiments resulting in the identification of 16,490 high confidence peaks. 95% of previously identified RARα binding sites were validated and good reproducibility was observed between biological repeat experiments (r^2=0.92) (**Figure 1**). These data sets allowed a comprehensive analysis of the genomic targets of RARα in Th1 cells.

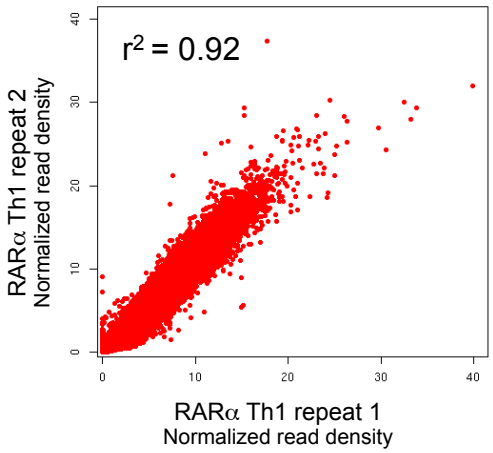

Figure 1. Reproducibility of Th1 RARα ChIP-seq data Global comparison of RARα ChIP-seq experiments between biological duplicates demonstrates reproducibility of RARα data. The coeffecient of determination is indicated in top left hand quadrant.

33 (70.2%) genes downregulated in dn*Rara* Th1 cells were bound by RARα vs. 53 (44.5%) upregulated genes (**Figure 2A**), confirming our earlier findings that RARα plays a dominant role in positive regulation of gene expression. We previously identified a number of Th1 defining genes whose expression was directly regulated by RARα, as well as RARα-mediated antagonism of genes that drive the Th17 lineage (Brown et al., 2015). To determine the extent to which RARα regulates the Th1 genome, we performed gene set enrichment analysis (GSEA) of transcriptional data from wild-type (WT) and dn*Rara* expressing Th1 cells, generated in **Chapter 2**. As well as dysregulation of the Th1-Th17 axis, GSEA revealed enrichment of genes preferentially expressed in alternative Th cell lineages, specifically Treg and Tfh cells, in dn*Rara* Th1 cells (**Figure 2B**).

To test for enrichment of RARα binding at T-helper cell specific genes we used publicly available transcriptome data for T-helper cell subsets to identify a set of T-helper cell specific genes. Th1 genes were enriched for RARα binding relative to signature genes associated with other $CD4^+$ T-cell subsets. In addition, genes from opposing T-helper cell subsets, in particular Treg genes, were enriched for RARα binding relative to a group of genes randomly selected across the genome (**Figure 2C**). The increased expression of genes associated with alternative T helper cell lineages in dn*Rara* Th1 cells, along with the presence of RARα at these loci, led us to hypothesize that RARα may occupy regions targeted by TFs of opposing lineages in order to inhibit TF activity at these loci. To test this, we determined the overlap of Foxp3 binding sites in Treg cells (Samstein et al., 2012), with RARα binding sites in Th1 cells.

We found that 1658 of the binding sites were shared between the two factors, representing 64% of Foxp3^{+} binding sites and 10% RARα binding sites (**Figure 2D**). These findings suggest that RARα may reciprocally regulate T helper cell developmental pathways by competitively antagonizing the action of transcription factors that instruct alternative cell fates. Furthermore, these findings lend insight into the increased frequency of Foxp3^{+} cells observed in dn*Rara* mice (Brown et al., 2015; Pino-Lagos et al., 2011). Collectively, these data indicate that RARα stabilises the Th1 phenotype through promotion of Th1 cell genes and repression of genes implicated in alternative T helper cell programs.

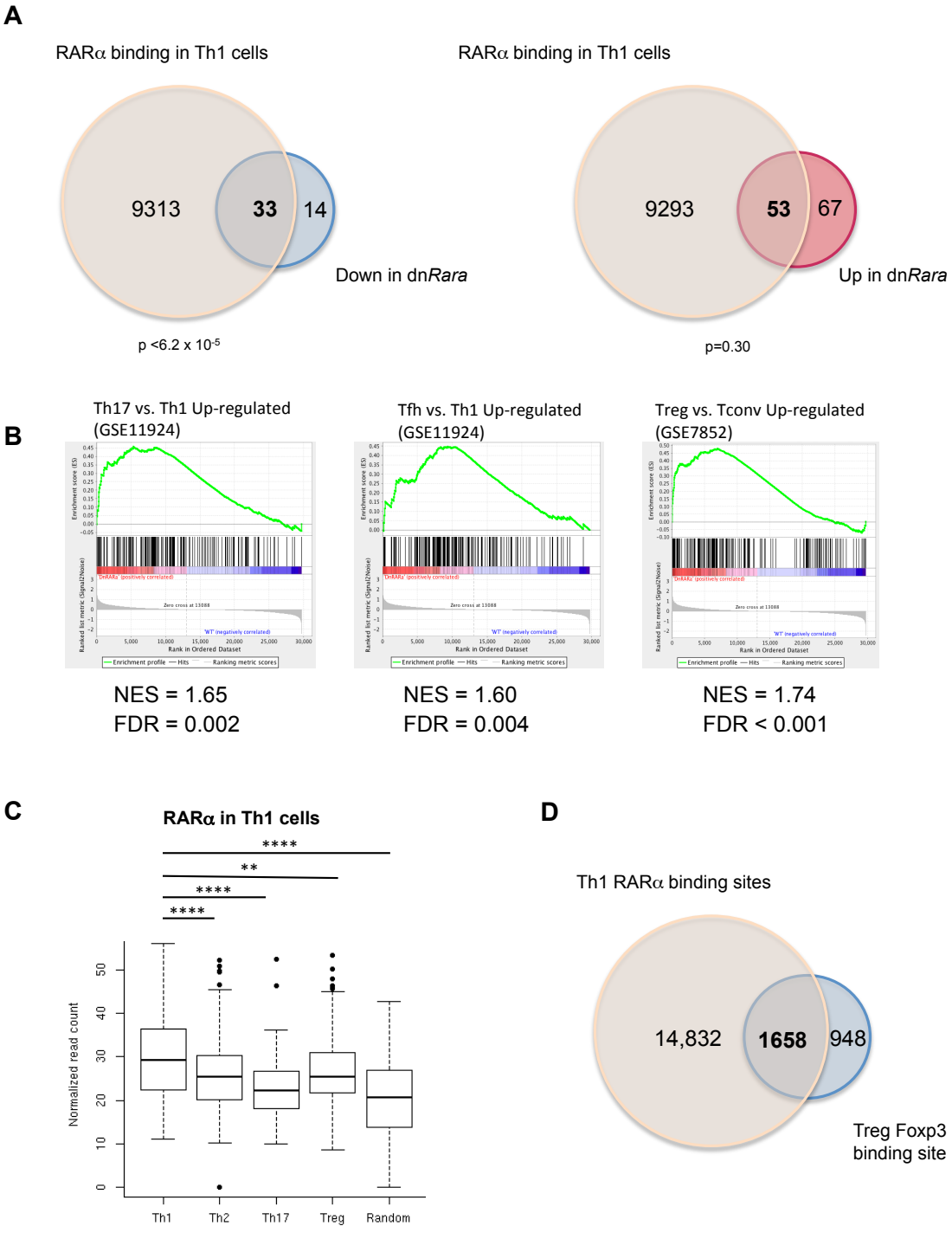

Figure 2. A global role for RARα in Th1 lineage stability

(A) Overlap between the set of genes bound by RARα and the genes up- or downregulated in dn*Rara* Th1 cells (fold change 1.5, FDR <0.05) **(B)** In each plot the genes from the array are sorted from left (upregulated in the respective comparison) to right (downregulated). The green plot indicates the cumulative GSEA score; black bars indicate the location of genes. **(C)** Th1-specific genes show enrichment of RARα binding. Box plots show median and quartiles of normalised RARa binding in Th1 cells at Th1-, Th2-, Th17- ,Treg-specific genes and 200 randomly selected (Random) genes (+/- 20kbp from the TSS). **(D)** RARα binding sites overlap with Foxp3 in Treg cells.

RARα is a feature of active enhancer regions

To further explore how RARα regulates the transcription of genes required for Th1 cell function and represses genes from opposing T cell lineages, we analysed the distribution of RARα bound regions in Th1 cells. Approximately half (51%) of RARα binding sites localised to promoter regions (**Figure 3A**). To determine the transcriptional effects of promoter-bound RARα, we performed ChIP-seq for the epigenetic marks H3K4me3 and H3K27me3, histone modifications associated with transcriptional activity, and repression respectively. These experiments revealed that RARα was largely associated with active promoters (**Figure 3B**). Surprisingly, comparison of H3K4me3 and H3K27me3 profiles at RARα bound Th1 promoters in wild-type and dn*Rara* Th1 cells did not reveal significant differences in deposition of these histone marks (**Figure 3B**).

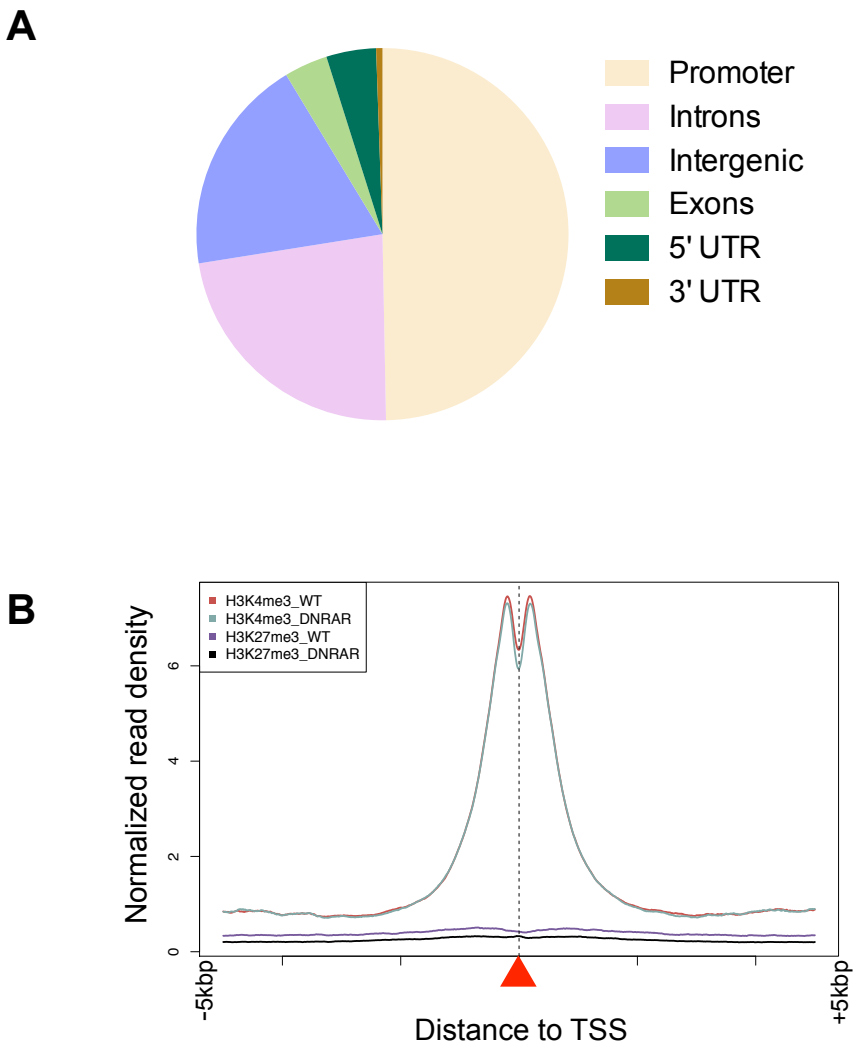

Figure 3. Genome-wide occupancy by RARα in Th1 cells
(A) Genomic distribution of 16,490 RARα binding sites in Th1 cells at promoter, intergenic (>4kbp TSS), and intragenic regions. (**B**) Normalised tag density profiles for H3K4me3 and H3K27me3 at RARα bound promoters (-4kbp to +500bp of transcriptional start site [TSS]) in dn*Rara* and WT Th1 cells. Plots show the normalized distribution of H3K27me3 or H3K4me3 at TSS (+/- 5 kbp)

The remaining RARα bound regions were located largely at introns and intergenic regions (**Figure 3A**), suggesting RARα might function at regulatory enhancer regions. Comparison of RARα binding with H3K4me1 and p300 outside of promoter regions (-4kbp to TSS and +500bp) demonstrated that RARα was largely associated with genomic regions marked by both H3K4me1 and p300, putative enhancer elements (**Figure 4**). Very few RARα bound sites were marked with H3K4me1 in the absence of p300, so called 'permissive' enhancers, indicating that RARα was either recruited to active enhancers or played a role in enhancer activation.

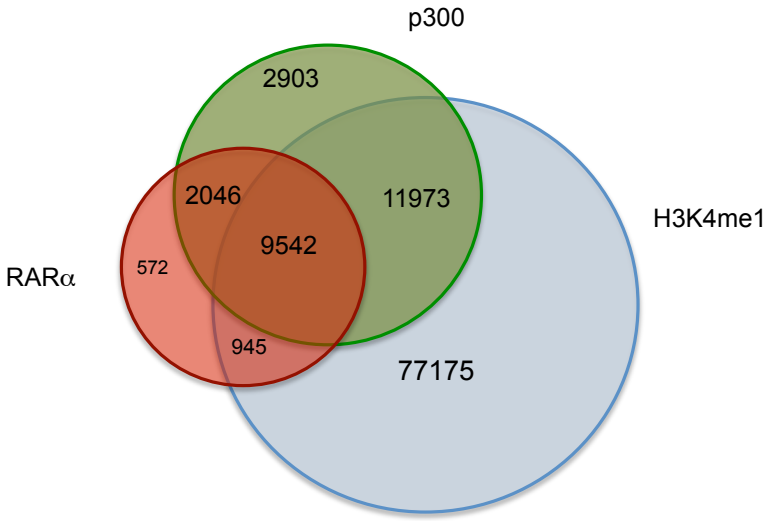

Figure 4. RARα binding and epigenetic modifications
Global mapping identifies concordance between H3K4me1, p300 and RARα in Th1 cells

To further evaluate the role of RARα in enhancer elements, we assessed the proportion of active enhancers bound by RARα in Th1 cells. Enhancer elements were operationally defined as regions enriched for H3K4me1 and p300 but depleted of H3K4me3 (Heintzman et al., 2007). Promoter regions were excluded from the analysis. From this analysis, we identified 10,511 putative enhancer elements. In embryonic stem cells, p300 marks both poised and active enhancers, with only the latter marked by H3K27ac (Rada-Iglesias et al., 2010). By contrast, in $CD4^+$ Th1 cells, nearly all (91%) enhancers defined by the presence of p300 were enriched for H3K27ac (**Figure 5**). This finding indicated that either p300 or H3K27ac was sufficient to distinguish an active enhancer in T cells.

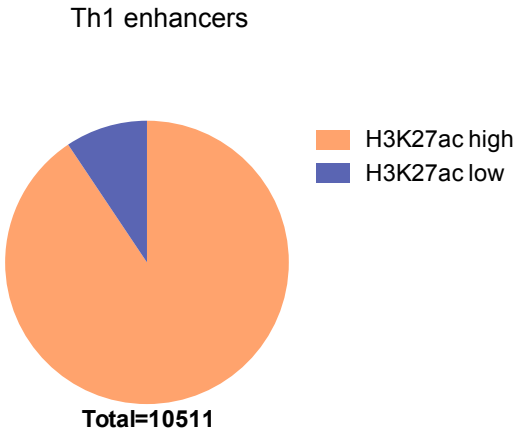

Figure 5. Defining Th1 enhancers
Enhancer regions defined by H3K4me1, p300 and low levels of H3K4me3 are marked by H3K27ac.

Strikingly, 3377 (32%) active enhancers were occupied by RARα (**Figure 6A**). This degree of overlap was similar to other Th1 lineage defining enhancers that have been implicated in enhancer activation (**Figure 6B**) (Vahedi et al., 2012). RARα binding at putative enhancers was observed at many key Th1 genes including *Tbx21*, *Stat4*, *Ifng* and *Irf8, Ifngr1, Il12rb2* (**Figure 6C**) many of which were previously shown to be downregulated in the absence of RA signaling (Brown et al., 2015).

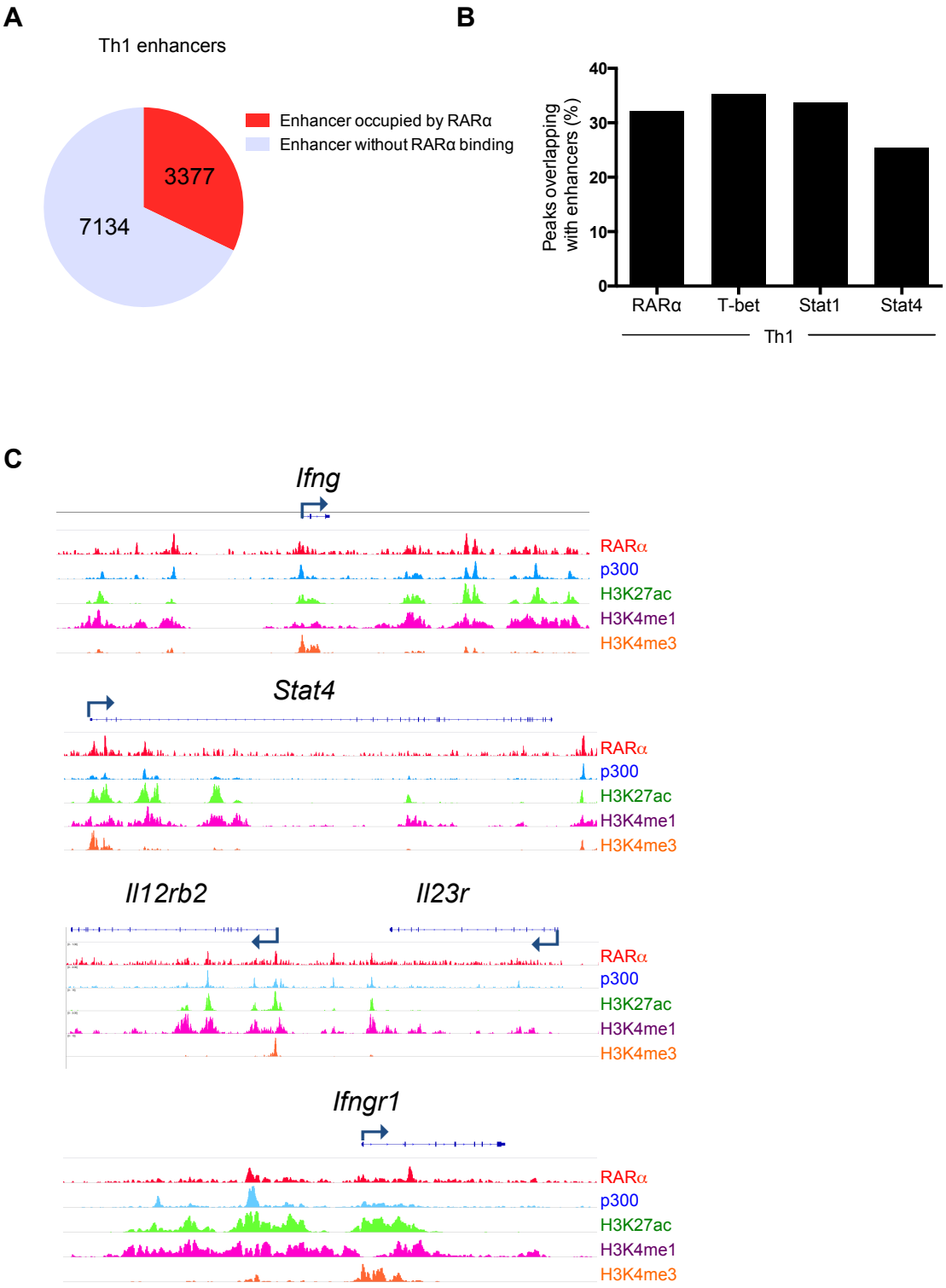

Figure 6. RARα is a key feature of the Th1 enhancer landscape
(A) Active enhancers are bound by RARα **(B)** Overlap of enhancer regions with RARα and Th1 lineage defining TFs **(C)** Chromatin signatures at sites of RARα binding. Tracks show RARα, H3K4me1, p300 and H3K27ac occupancy across *Ifng*, *Stat4*, *Il12rb2* and *Ifngr1* in Th1 cells

Analysis of Th1 enhancers demonstrated that RARα bound enhancers were enriched for p300 and H3K27ac relative to enhancers lacking RARα, indicating a potential role for RARα in enhancer activity (**Figure 7**). These data suggest that in Th1 cells, RARα binds at enhancers where it acts to regulate gene transcription.

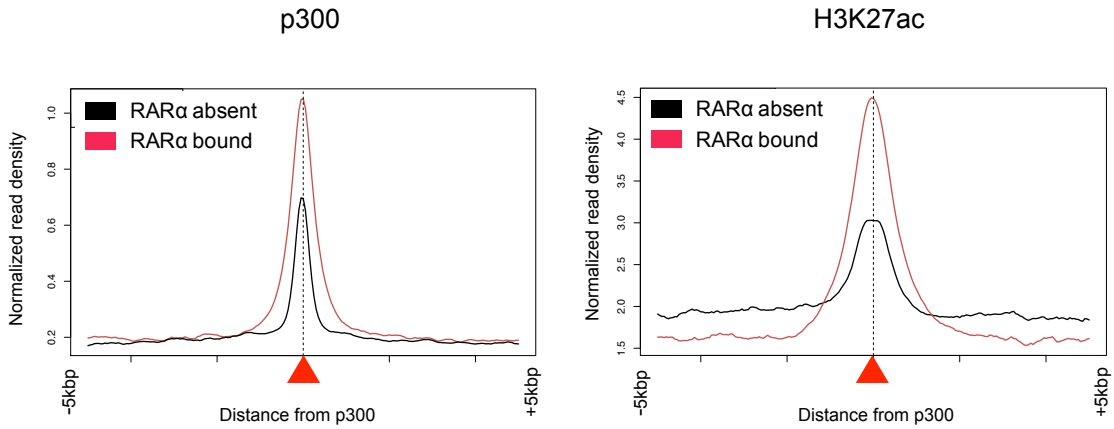

Figure 7. RARα binding at enhancers predicts activation
Compiled tag density profiles for p300 and H3K27ac at RARα bound enhancers vs. RARα negative enhancers in Th1 cells. The presence of RARα at enhancers is associated with increased p300 and H3K27ac.

Enhancer bound RARα regulates transcription

To determine the effects of RARα at enhancers on gene expression, we performed overlap analyses comparing the overlap of DEGs in dn*Rara* Th1 cells with genes where RARα was bound only at the promoter region or an enhancer or both. Consistent with the histone profiles, genes harboring RARα solely at promoters did not exhibit significant changes in gene expression (**Figure 8**). In contrast, genes bound by RARα at enhancers were significantly enriched among genes positively and negatively regulated by RA/RARα activity in Th1 cells. Collectively, our findings reveal that RARα is enriched at Th1 enhancers and its presence correlates with functional activity.

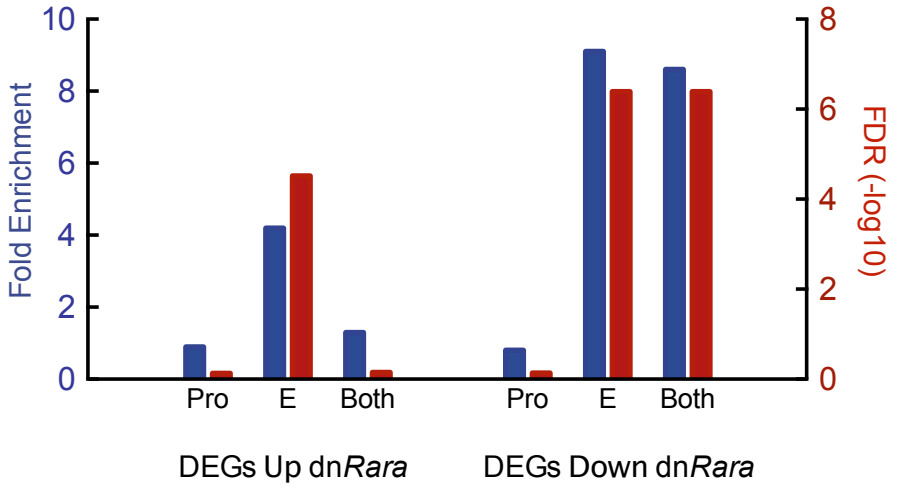

Figure 8. **RARα activity at enhancers regulates gene expression**
Enrichment (blue bars) and significance (red bars) of dnR*ara* differentially expressed genes in genes bound by RARα at the promoter (P), enhancer (E) or both. Fisher's exact test.

RARα signaling is required for Th1 enhancer activation

In our previous study of selected RARα-bound enhancers, p300 occupancy was dependent on RA signaling; this suggested that RA/RARα regulation of enhancer functionality may represent a pervasive mechanism by which RA/RARα positively regulates gene expression. To explore the functional consequences of RARα at active enhancer elements, we utilized p300 ChIP-seq data from WT and dn*Rara* expressing Th1 cells. Good reproducibility was observed for p300 binding in biological repeat experiments (**Figure 9**).

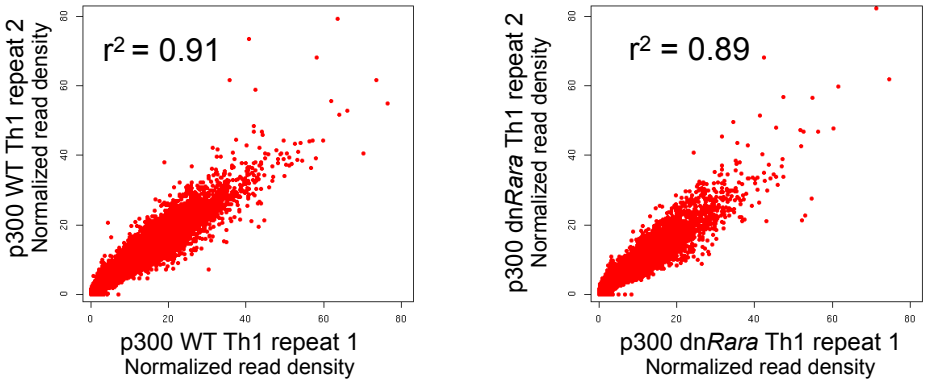

Figure 9. Reproducibility of p300 ChIP-seq data
Global comparison of p300 ChIP-seq experiments between biological duplicates of WT and dn*Rara* Th1 cells demonstrates reproducibility of p300 data. The coeffecient of determination is indicated in top left hand quadrant.

dnRARα lacks the AF-2 domain, which is the site of interaction with p300. We found that among 3377 RARα bound Th1 enhancers, 20% (698) are dependent on RA signaling with a dramatic inhibition of enhancer activity as evidenced by loss of p300 and H3K27ac (**Figure 10A** and **10B**). Only 30 RARα enhancer regions showed increased p300 in the absence of RA signaling. Although H3K4me3 is depleted at enhancers relative to promoters, its presence is associated with enhancer activity (Wang et al., 2008). We also observed reductions in H3K4me3 at RARα bound enhancers in dn*Rara* Th1 cells (**Figure 10C**), indicating reduced transcription from these sites. Intensity of H3K4me1 at the p300 peak summit was similar between dn*Rara* and WT Th1 cells; although the breadth of the peak was reduced in the absence of RA signaling, the unchanged peak intensity suggests that RARα activity is not required for deposition of H3K4me1 (**Figure 10D**). Loss of RA signaling did not result in increased levels of the repressive mark, indicating that regulation of enhancer activity by RA is through p300 recruitment, not removal of H3K27me3 (**Figure 10C**). Thus, RARα is associated with enhancers where its ability to recruit coactivators is required for enhancer activation.

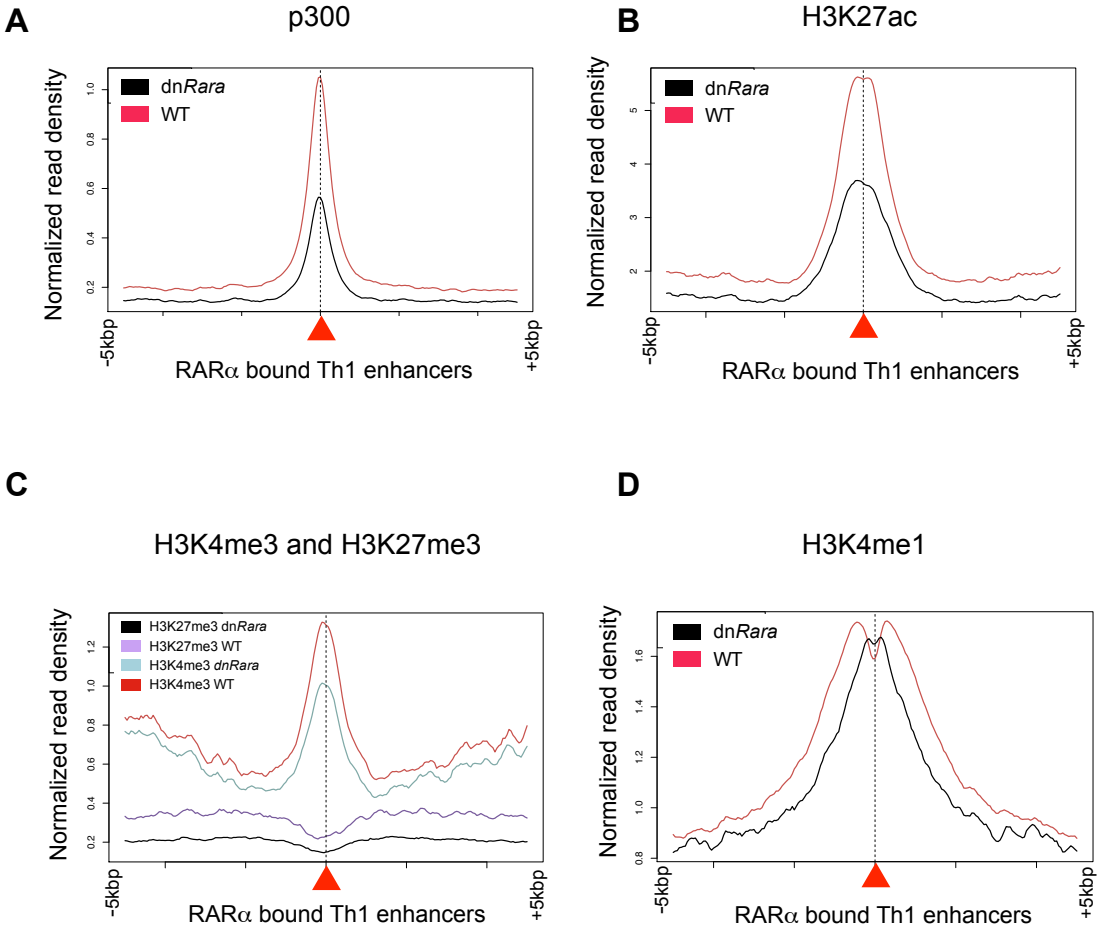

Figure 10. RARα signaling is critical for active enhancers in Th1 cells
Tag density profiles for **(A)** p300, **(B)** H3K27ac, **(C)** H3K27me3 and **(D)** H3K4me1 at RARα bound enhancer regions in dn*Rara* and WT Th1 cells demonstrate that RARα bound enhancers are dependent on RA signaling for p300 recruitment and functional activity. Removal of H3K27me3 from enhancers is not regulated by RARα signaling **(C)**.

RARα is enriched at lineage defining super-enhancers

Super-enhancers (SEs) have been suggested to be the key determinants of lineage identity. Given the essential role for RARα in maintaining Th1 cell fate, we assessed the proportion of Th1 SEs with RARα occupancy using publicly available data for SE regions in T helper cell subsets (Vahedi et al., 2015). Remarkably, we found that RARα binding was present at 85% of Th1 SE regions (**Figure 11A**), with a similar level of enrichment at Th1 lineage-specific SEs (77%) (**Figure 11B**). The observed increased frequency of RARα occupancy at SEs vs typical enhancers may have reflected the increased size of SE regions: the genomic regions of SEs are approximately an order of magnitude greater than that of a typical enhancer element (Whyte et al., 2013). To assess the specificity of this enrichment, we evaluated the occupancy of RARα at a comparable region of the genome. For this purpose we examined, within Th1 cells, the genomic elements which have super-enhancer structure specifically in Th2 cells but not Th1 cells (Vahedi et al., 2015). We did not observe an increase in RARα occupancy at these regions (**Figure 11C**), confirming the specificity of RARα binding to Th1 SEs. To determine the contribution of RARα in generating super-enhancer structures, we compared p300 profiles in WT and dn*Rara* Th1 cells. Deficiency in RARα signaling resulted in a significant reduction in p300 marks at SEs bound by RARα but not RARα depleted super-enhancers (**Figure 11D**), arguing for a direct role of RARα in generating super-enhancers. Collectively, these data indicate that RARα signaling is enriched at cell-type specific SEs and is essential for establishing functional super enhancer regions.

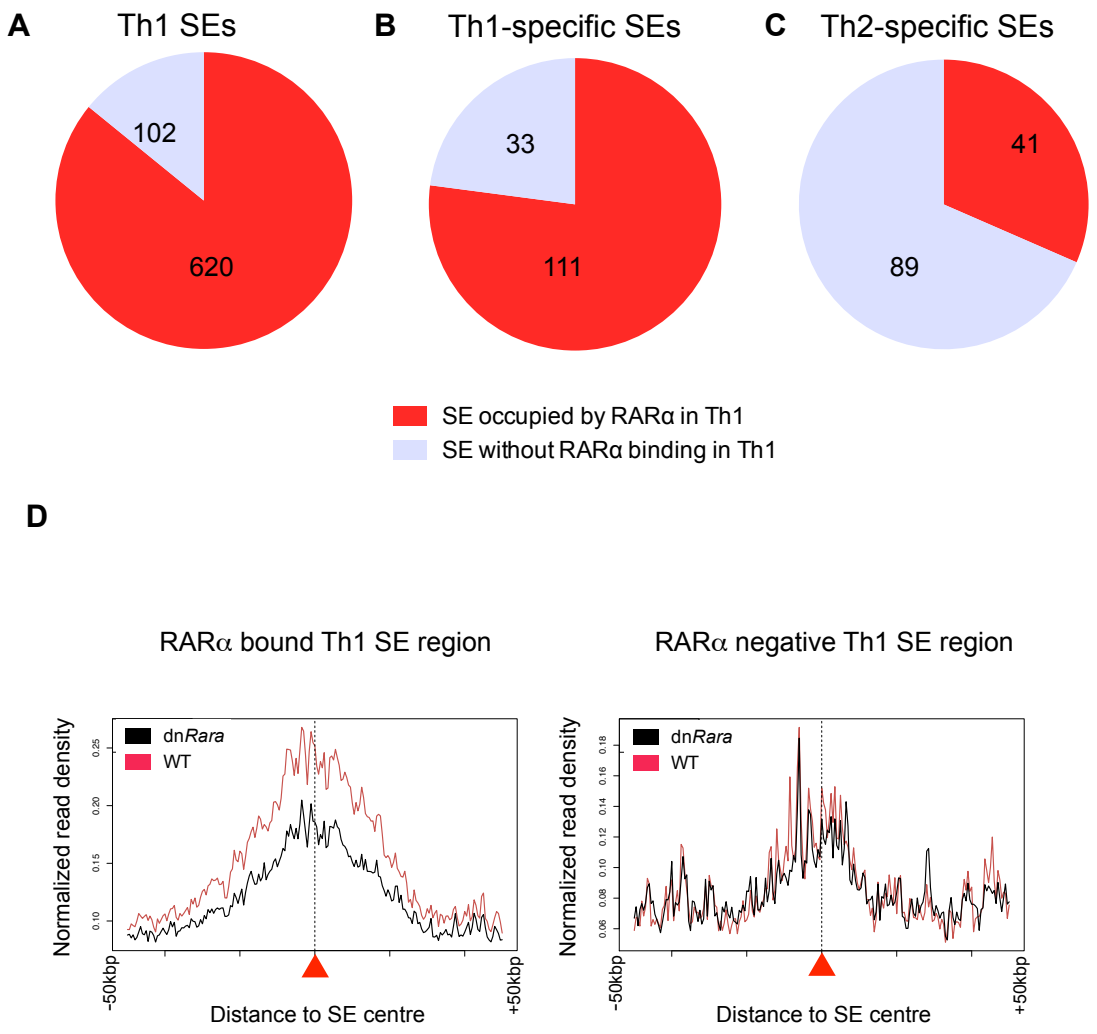

Figure 11. RARα is enriched at Th1-specific super-enhancers and is critical for the generation super-enhancer architecture.
Piecharts showing (**A**) the proportion of Th1 super-enhancers (SEs) bound by RARα in Th1 cells, (**B**) the proportion of Th1-specific SEs bound by RARα in Th1 cells or (**C**) the proportion of regions with SE architecture in Th2 cells bound by RARα in Th1 cells. SE regions are defined in Vahedi et al. 2014. (**D**) Tag density plots showing p300 binding patterns across Th1 SE regions in dn*Rara* and WT Th1 cells at RARα bound Th1 SEs (left panel) or RARα negative Th1 SEs (right panel).

RARα is the dominant chromatin modifier at enhancer elements

In a recent study, Th1 lineage-defining STAT proteins, STAT1 and STAT4, were shown to associate with enhancers and regulate enhancer activity with a minor role for T-bet. Given that these TFs show similar degrees of overlap with enhancer regions, we wondered whether these factors were binding at the same enhancers and regulating enhancer activity in a co-operative manner. To better understand the relationship between RARα and other Th1 associated transcription factors, we evaluated the co-occupancy of RARα and lineage specific TFs using publicly available ChIP-seq data sets to compare the binding profiles of T-bet, STAT1 and STAT4 in Th1 cells at enhancers. There was extensive overlap between individual RARα binding sites and those of key Th1 cell regulators (**Figure 12A**), with co-occupancy noted at enhancers of key Th1 genes (**Figure 12B**).

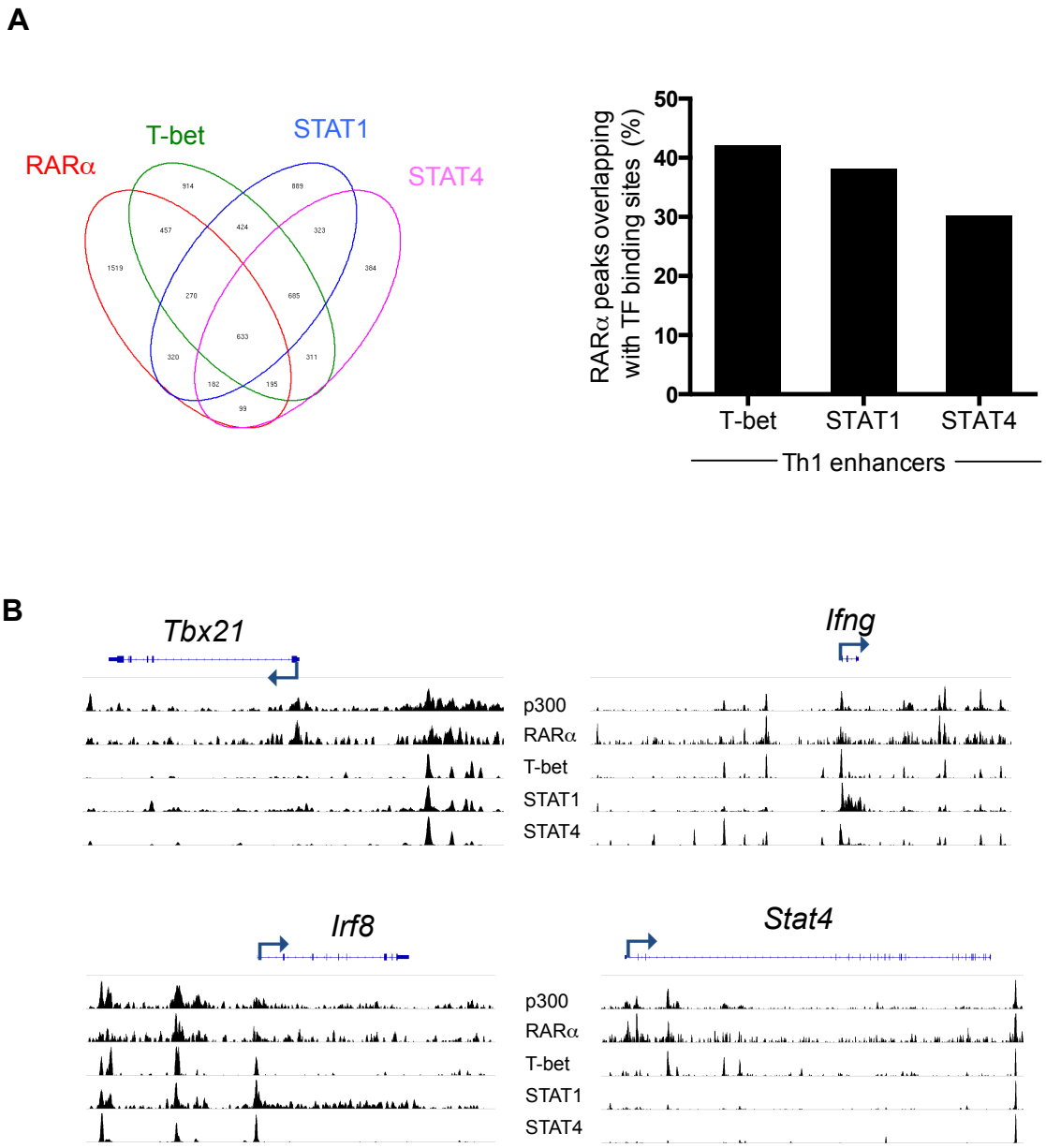

Figure 12. Co-localization of RARα and key Th1 transcription factors at active enhancers in Th1 cells

(A) The numbers of peaks identified for RARα bound enhancers that co-localize with T-bet, STAT4 and STAT1 bound enhancers in Th1 cells (left panel). Numbers of peaks identified for overlapping proteins as a percentage of the total RARα-bound enhancer peaks identified (right panel). **(B)** Co-occupancy of RARα, p300, T-bet, STAT1 and STAT4 at enhancers of Th1-defining genes in WT Th1 cells

To determine the relative contribution of each of these factors on recruitment of p300 to enhancers, we compared p300 intensity at enhancers with different patterns of transcription factor occupancy. Similar levels of p300 enrichment were observed at enhancers bound solely by RARα, T-bet, STAT4 or STAT1 (**Figure 13**), indicating that each of these factors can play a role in p300 recruitment. Interestingly, RARα had a far greater effect on H3K27ac deposition at enhancers than the lineage associated transcription factors, despite equivalent levels of p300, indicating that RARα may also regulate histone acetylation through recruitment of other co-activators with histone acetyltransferase activity, such as CBP. H3K27ac correlates with transcriptional activity at enhancer regions, suggesting that the functional strength of RARα bound enhancers is greater than enhancers occupied solely by T-bet, STAT4 or STAT1. An increase in p300 and H3K27ac intensity was observed when RARα was co-bound with any other of the lineage determining transcription factors indicating coordinate activation of enhancers by signal dependent and lineage specific transcription factors (**Figure 13**).

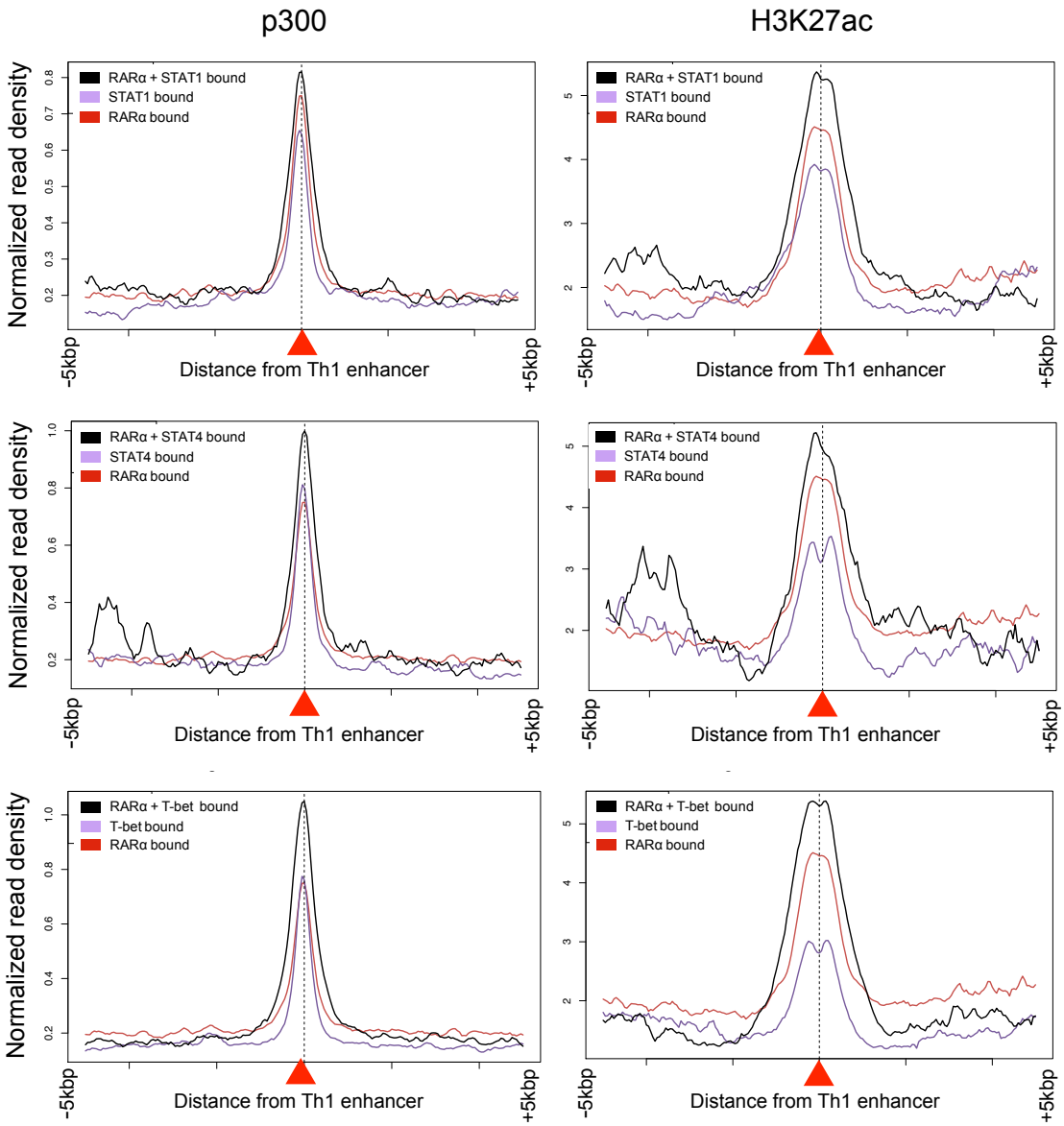

Figure 13. Regulation of p300 binding by RARα and key Th1 transcription factors.
Compiled tag density profiles for p300 (left panel) and H3K27ac (right panel) at Th1 enhancers uniquely bound by RARα, STAT1, STAT4 or T-bet; or RARα in combination with one of STAT1, STAT4 or T-bet. RARα has comparable effects on p300 recruitment with other Th1 lineage-determining transcription factors (LDTFs) but greater effects on H3K27ac. Combinatorial effects of RARα and Th1 LDTFs on p300 recruitment and H3K27ac are observed.

To determine the relative contribution of these factors to p300 recruitment, we performed a linear regression analysis to predict p300 intensity from the presence of these transcription factors. As expected, all four transcription factors had statistically significant predictive ability (**Figure 14**). Strikingly, the presence of RARα was the greatest predictor of p300 intensity. Intriguingly, the analysis revealed a synergistic relationship between T-bet and RARα on p300 recruitment, which was not observed for any other pairwise combinations of transcription factors. Collectively, these data indicate that an RA signal at RARα bound enhancers is an additional mechanism by which enhancers can become activated and suggest that RARα is the dominant regulator of p300 at enhancers.

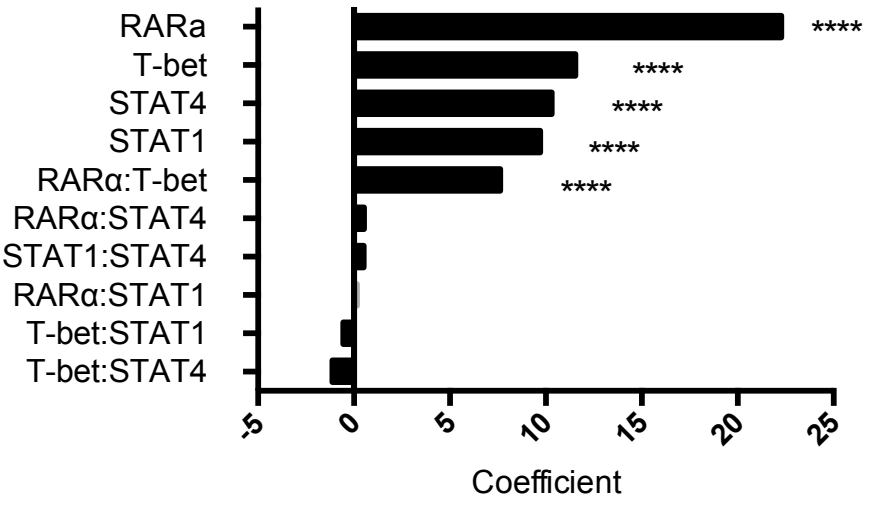

Figure 14. RARα is the dominant regulator of p300 recruitment at Th1 enhancers
A multiple linear regression analysis was calculated to determine the predictive effects of RARα and Th1 transcription factors on p300 binding at Th1 enhancers. Coefficient reflects the size of the effect. The presence of RARα and T-bet together had a greater effect on p300 intensity than would be expected from the individual effects of these factors. **** p <0.0001

RARα is recruited to cell-type specific enhancers during differentiation

Enhancers are cell-type specific and developmental stage specific. Yet it has also been proposed that RARα is constitutively bound to its targets and in the absence of retinoids, co-repressors are recruited to the receptor to prevent transcription. To determine whether RARα was bound at permissive enhancers in naïve T cells that become active during Th1 differentiation, or whether RARα is recruited to enhancers, we compared binding of RARα in naïve $CD4^+$ T cells, Th1 cells and Th17 cells. Comparison with Th17 cells allowed assessment of RARα binding in response to TCR activation, independent of the cytokine milieu. p300 ChIP-seq in Th17 cells was also performed to identify Th17 cell enhancers. We identified 13,014 statistically significant RARα peaks in naïve $CD4^+$ T cells and 12,426 in Th17 cells with good reproducibility between biological repeat experiments (r^2 = 0.86) (**Figure 15**).

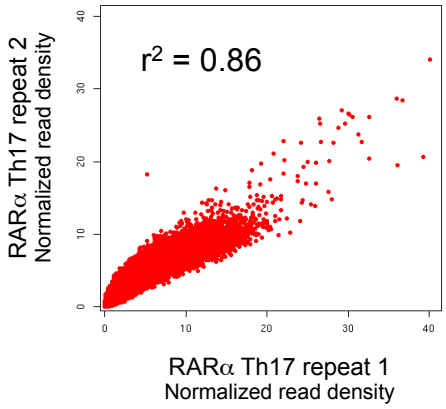

Figure 15. Reproducibility of RARα ChIP-seq data in Th17 cells

Global comparison of RARα ChIP-seq experiments between biological duplicates demonstrates reproducibility of RARα data. The coeffecient of determination is indicated in top left hand quadrant.

Of these, 5552 RARα peaks (33% of Th1 and 45% of Th17) were common to naïve, Th1 and Th17 cells (**Figure 16**). We observed a shift in RARα binding during $CD4^+$ T cell differentiation with 6217 peaks exclusively bound in Th1 cells, representing 37% of RARα bound Th1 sites, and 3,728 novel peaks in Th17 cells (30% of total Th17 peaks) (**Figure 16**). Only 1965 (16%) differentiation-induced RARα bound elements were shared between Th1 and Th17 cells indicating that distinct environmental cues direct lineage specific RARα binding.

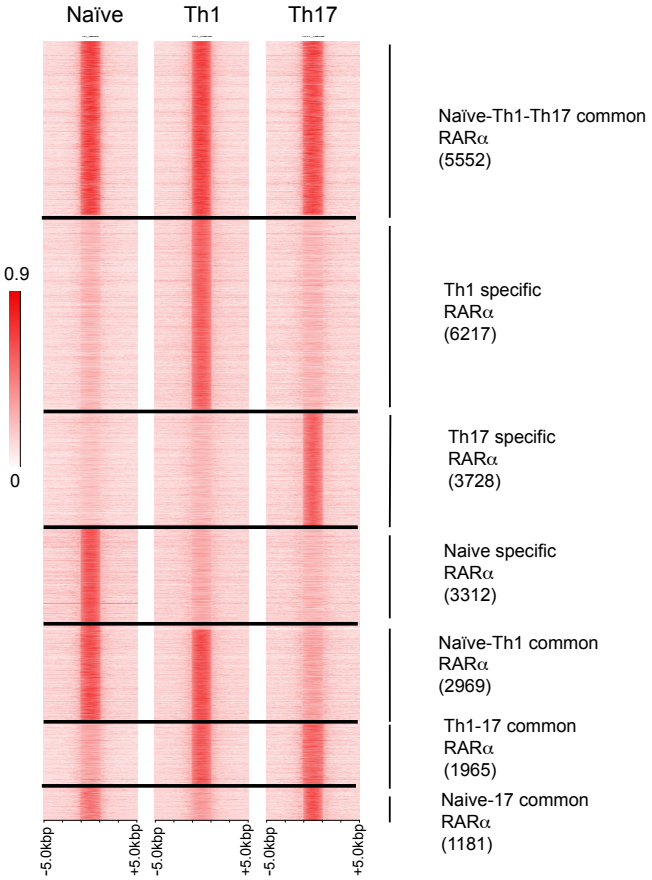

Figure 16. RARα binds to cell-specific regions during naïve $CD4^+$ T-cell differentiation
Heatmaps, based on ChIP-seq data, showing RARα binding in naïve, Th1 and Th17 cells. The plot in each column represents the pattern of RARα binding centred on the RARα-bound sites in each cell type.

Comparison of cell-specific RARα binding peaks revealed that RARα binding in response to differentiation was largely directed to regulatory regions (**Figure 17**), consistent with cell-type-specific actions of RARα. 3071 (49%) of Th1 specific peaks and 769 (21%) of Th17 specific peaks overlap Th1 and Th17 enhancers respectively. For this analysis, putative enhancers were defined as p300 binding peaks outside of promoters.

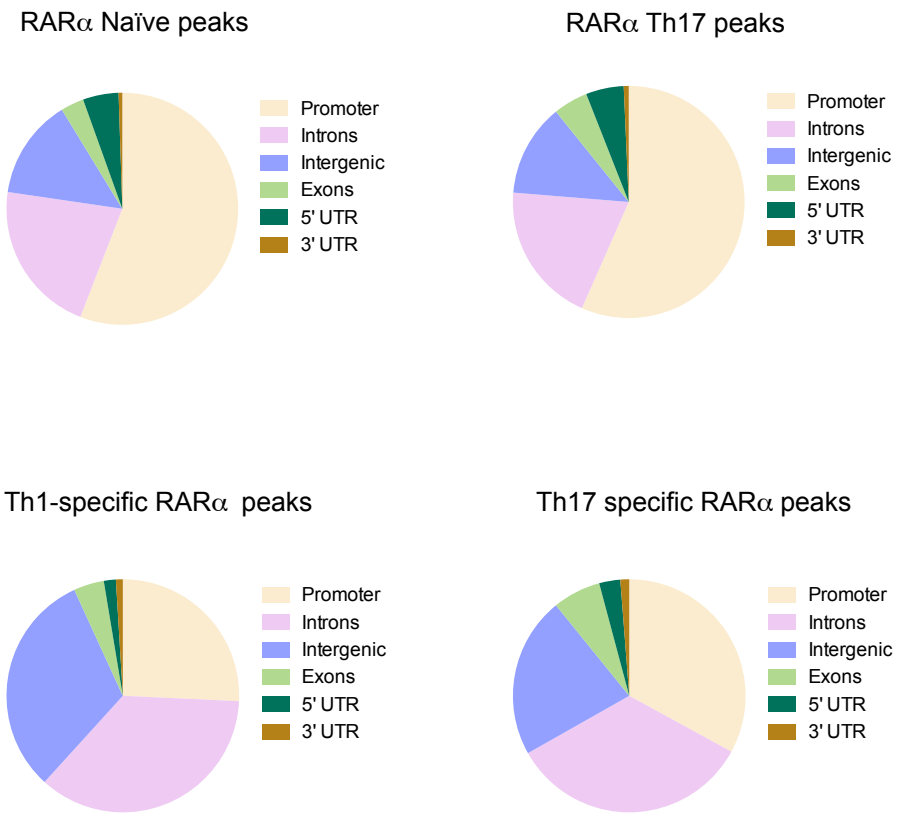

Figure 17. Cell-type specific binding peaks are enriched at regulatory regions
Pie chart of the genome-wide distribution of RARα peaks in naïve and Th17 cells (upper panel), and cell-type specific peaks in Th1 and Th17 cells (lower panel), based on Ref-seq. Cell-type specific peaks are enriched at intergenic regions and introns.

Analysis of T helper cell signature genes demonstrated lineage specific patterns of RARα binding with overlap of RARα at the relevant lineage enhancer (**Figure 18A**). Genes selectively expressed in Th1 cells were significantly enriched in genes harbouring a Th1-specific RARα binding site (**Figure 18B**). Similarly, analysis of genes containing Th17-specific RARα bound loci revealed significant overlap with a literature-curated set of Th17 relevant genes (**Figure 18B)**. Collectively, these data show that in response to cytokine-directed differentiation, RARα selectively binds to elements within genes preferentially expressed in the relevant lineage. This indicates that RARα regulation of lineage specific genes occurs in response to extrinsic cues that drive differentiation of naïve $CD4^+$ T cells towards a specific lineage.

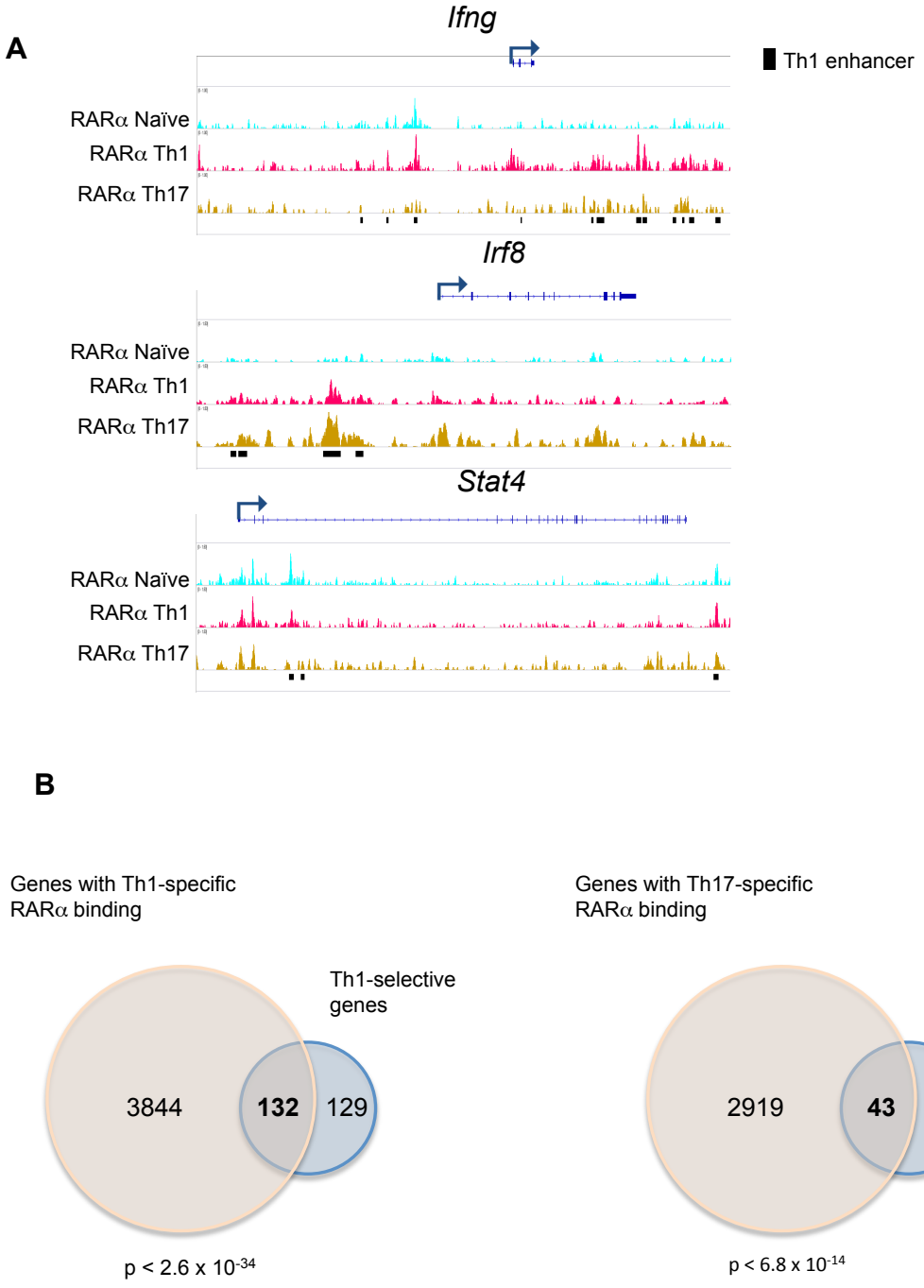

Figure 18. RARα binds overlapping and distinct T helper cell enhancers and lineage-defining genes

(A) Gene track represents RARα binding in WT naïve, Th1 and Th17 cells in *Ifng*, *Irf8* and *Stat4* locus. **(B)** Overlap of signature Th1 (left panel) and Th17 (right panel) genes harbouring cell-type-specific RARα peaks with T helper cell signature genes. p values were calculated using a hypergeometric probability test.

Functional analysis of lineage specific RARα bound regions

To gain further insight into the lineage specific pathways targeted by RARα, likely to be relevant to Th1 function, we performed pathway analyses on the genes associated with unique RARα binding sites in Th1 cells. For this, we used GO and KEGG databases as well as MSigDB (Broad Institute). As expected, genes involved in cytokine signaling and lineage specification were highly enriched for genes containing Th1-specific RARα peaks. Strikingly, genes associated with regulation of metabolic processes, including OXPHOS and mTOR signaling, were significantly enriched in genes harbouring Th1-specific RARα-binding sites (**Figure 19**). It is increasingly appreciated that these metabolic pathways are critical for successful effector T-cell responses. A number of signaling pathways including TCR, cytokine and complement signaling have been shown to regulate the expression of key metabolic genes. As well as regulation of lineage-defining transcription factors, cytokines and their receptors, it now appears that RA/RARα also regulates metabolic pathways. Intermediaries in metabolic pathways are key cofactors required for epigenetic modifications. In addition, pathway analysis identified enrichment of genes involved in H3K9 demethylation. Thus RA/RARα may regulate enhancers by synthesis as well as recruitment of epigenetic modifiers.

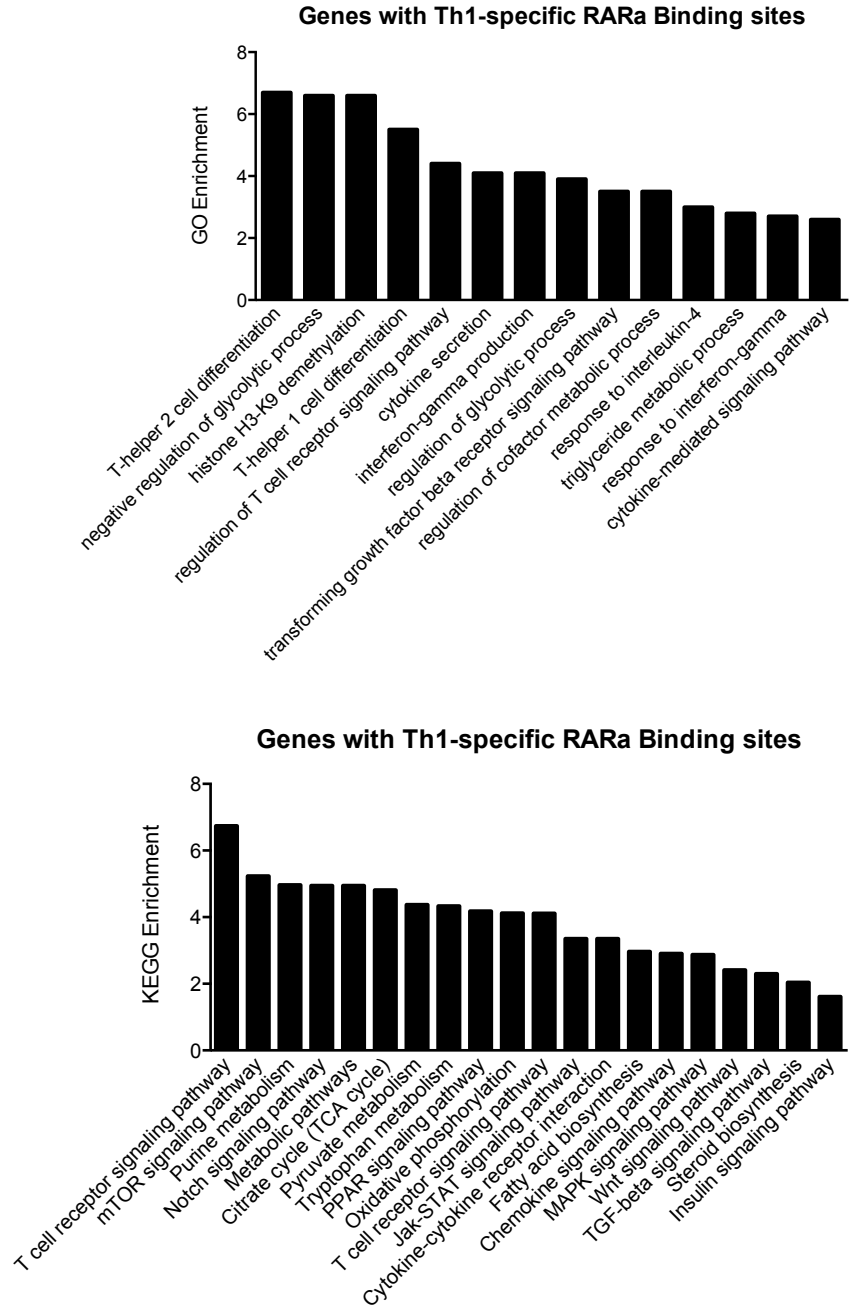

Figure 19. Functional analysis of Th1-specific RARα binding regions
Pathway analysis of genes associated with Th1-specific RARα peaks using GO and KEGG databases.

RA/RARα regulates Th17 differentiation

The finding of unique binding sites for RARα in Th17 cells at Th17 signature genes was also of interest, as the role of RA in these cells has been one of continuing controversy. Our previous study (**Chapter 2**) along with a number of published studies of RA and Th17 cells suggest an inhibitory role for RA/RARα on the Th17 program. To determine the functional significance of RARα binding at these loci we polarized dn*Rara* naïve $CD4^+$ cells under Th17 conditions and analyzed the expression of IL-17 and RORγt. Surprisingly, in contrast to the repression of the Th17 phenotype by RARα in Th1 cells, we did not observe enhanced Th17 responses in Th17-polarised dn*Rara* $CD4^+$ T cells. Preliminary data showed a loss of Th17 phenotype at day 6 of culture, indicating a similar requirement for RA in maintenance of Th17 cell fate (**Figure 20A**). A third of Th17 enhancers, defined as p300 peaks outside of promoters, were bound by RARα in WT Th17 cells at day 3 of differentiation (**Figure 20B**). At day 5, a similar number (2276) of p300 binding sites were occupied by RARα reflecting 17% of total enhancers at this timepoint (**Figure 20B)**. Similar to our findings in Th1 cells, RARα was associated with increased p300 intensity at enhancer regions (**Figure 20C)**. Transcriptional profiling is required to confirm the significance of RARα bound Th17 enhancers, but preliminary results suggest an important role for RARα in Th17 cell fate. Collectively, these findings show that RARα binds to overlapping and distinct genomic regions in T helper cell subsets, and suggest that RARα regulates T helper cell fate by targeting enhancers at genes that define lineage and regulate cell function.

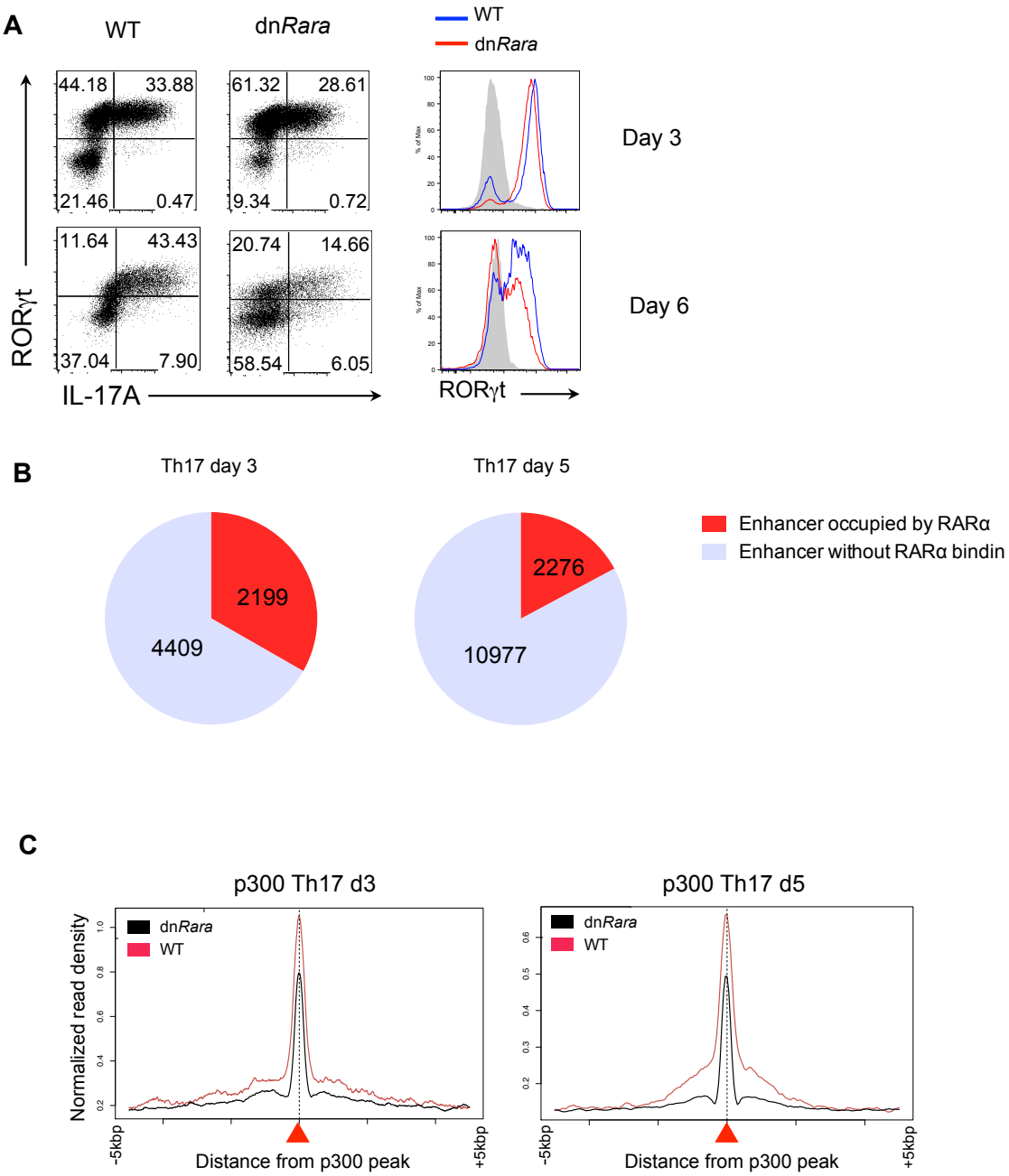

Figure 20. RARα regulates Th17 differentiation

(A) Naïve CD4$^+$ T cells from dnRara or WT mice were cultured under Th17 conditions. Intracellular expression of IL17-A and RORγt expression was analysed at the indicated timepoints following stimulation with PMA and ionomycin. Numbers in quadrants represent percent cells in each. Grey histograms indicate isotype control antibody. **(B)** Piecharts representing the proportion of Th17 enhancers that are bound by RARα in Th17 cells at day 3 or 5 of culture. Regions of p300 binding outside of promoters were considered to be enhancer elements. **(C)** Tag density profiles of p300 binding in dn*Rara* or WT Th17 cells taken on day 3 (left panel) or day 5 (right panel) of culture.

Lineage determining transcription factors might recruit RARα for enhancer activation

RARα is broadly expressed by T helper cell subsets as well as non-T-cell lineages. However, the data above demonstrate cell-type-specific binding of RARα. The lineage specificity of differentiation-induced RARα binding in Th1 and Th17 cells suggested that cell context dependent effects of RARα on gene expression resulted from recruitment of RARα to lineage specific genes in response to extrinsic cues or intrinsic factors that are induced during lineage specification. A recent study identified RARα as part of a transcription factor complex that was bound in *trans* at oestrogen dependent enhancers. We hypothesized that RARα may play an analogous role in Th1 cells, where it may be recruited to cell-specific enhancer by other Th1 defining transcription factors. In order to identify potential pioneer factors or co-factors that may facilitate RARα binding, we studied the DNA sequences for regions bound by RARα specifically in Th1 cells, comparing enhancer with non-enhancer regions. Transcription factors with motif enrichment in Th1-specific RARα-bound enhancers included the 'master regulator' T-bet as well as Ets1, Runx and an activator protein 1 (AP-1) which may represent binding of a number of factors including BATF, Fos or Jun (**Figure 21**).

How RARα is selectively recruited to novel enhancers in Th1 and Th17 cells remains unclear. Two possible models exist: either pioneer factors at Th1 enhancers alter the chromatin accessibility of these regions and allow binding of RARα, or, lineage-specific factors bound at Th1 enhancer regions recruit RARα through their trans activation domains. Further analysis of the cell-type-specific

RARα bound enhancers are required to resolve these possibilities. Given the overlap of T-bet and RARα binding at enhancers (>40%) and the synergistic effect of RARα and T-bet on p300 recruitment, T-bet may play a key role in recruiting RARα to enhancers.

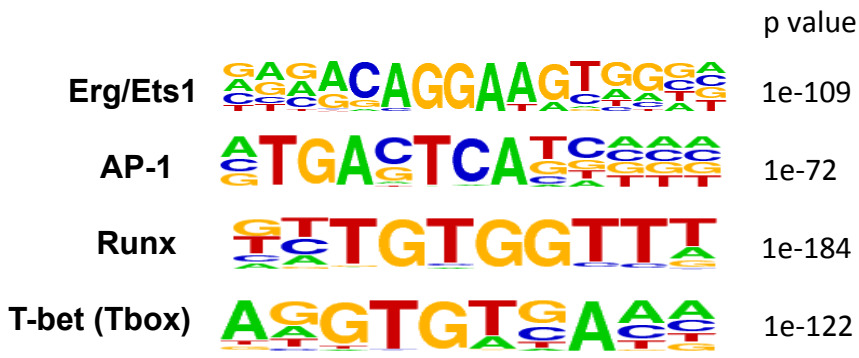

Figure 21. Identification of putative pioneer factors and RARα cofactors at Th1 enhancers
Examples of motifs within Th1-specific RARα-bound enhancers for transcription factors which may serve as pioneer factors or binding partners for RARα

Discussion

The molecular mechanisms responsible for orchestrating genome wide transcriptional responses, and epigenetic modifications during T helper cell differentiation, are not fully understood. Enhancers play a key role in directing cell fate through the regulation of lineage specifying genes, and there has been much interest in identifying the factors that link extracellular signals with changes at the chromatin level. The studies reported in Chapter 2 suggested that RA signaling in differentiating Th1 cells is able to regulate enhancer functionality. Here we set out to examine the extent to which RA/RARα regulates the Th1 cistrome. Two interesting conclusions emerge. One is that the RA/RARα axis regulates Th1 cell phenotype through multiple transcriptional pathways, not limited to a few classical transcription factors and cytokines. The other conclusion is that, although multiple transcription factors regulate the transition of Th1 enhancers to an active state, RA is dominant. Our data establish the RA/RARα signaling axis as a global regulator of the Th1 program.

Furthermore, the role of RA/RARα extends beyond Th1 cells. Mapping of RARα binding in naïve and differentiated T helper cells suggest that distinct transcriptional networks in differentiating $CD4^+$ T cells facilitate binding of RARα to subset specific enhancers. Comparison of RARα regulation of enhancers at different stages of differentiation and across $CD4^+$ T cell subsets identified lineage specific actions of RARα at genes that control cell identity. These studies reveal a molecular basis for a broader role for RARα in the regulation of cell fate.

RARα occupancy is a feature of functional enhancers in Th1 cells

Our findings suggest that the presence of RARα at enhancers predicts enhancer activation and that RARα signaling is critical for enhancer functionality. Signaling through RARα allows binding of p300 to the AF-2 activating domain of the RAR. Th1 cells expressing dnRARα that lacks the AF-2 domain had reduced levels of p300 and H3K27ac at RARα-bound enhancers, indicating a direct role for RARα in p300 recruitment. RARα may have other roles beyond p300 recruitment. Although similar levels of p300 occupancy were observed between RARα and STAT bound enhancers, RARα bound enhancers had far greater levels of H3K27ac. Although binding does not necessarily imply transcriptional changes, H3K27ac is predictive of enhancer functionality. The p300-independent effect of RARα may be because RARα interacts with a number of co-activators, including the histone acetyltransferase CBP (Kamei, 1996). In addition to recruitment of HATs, another mechanism by which RARα may regulate enhancers is illustrated by a recent report of RAR dependent recruitment of the DNA-dependent protein kinase, (DNA-PKc) which is required for ligand-induced activation of enhancer transcription (Liu et al., 2014).

Even more striking than the proportion of enhancers binding RARα was its highly enriched binding at super-enhancer regions. 86% of these exhibited RARα binding. Furthermore, we can infer a direct role for RARα in the regulation of super-enhancer activity from the finding that loss of p300 binding in dn*Rara* Th1 cells was only observed at RARα-bound super-enhancers.

The experiments in this chapter show that RARα signaling serves as a mechanism to promote recruitment of coactivators that increase enhancer activation and transcription.

Retinoic acid is a global regulator of T helper cell stability

Our findings build on previous findings in Chapter 2 that RARα reinforces Th1 stability by repressing genes associated with Th17 lineage. We now show that RARα additionally contributes to Th1 stability by directly controlling the expression of signature genes of other T helper cell lineages, including Tfh and Treg cells. Specifically, we have shown that in Th1 cells, RARα is bound at a significant proportion of the regions occupied by Foxp3 in Tregs. It will be important to identify the in vivo environments in which RA signaling regulates the conversion of Th1 cells to Treg or Tfh cells. A recent study showed that a single naïve T cell clone can give rise to a variety of different T helper cell types (Becattini et al., 2015). One possibility is that gradients in RA concentration, generated as a consequence of the distance of a proliferating cell from the source of RA, determines whether a differentiating cell maintains its original specification or adopts an alternative cell fate. Clearly flexibility in the $CD4^+$ T cell response can be advantageous. For instance a Th1 to Treg switch would allow the development of Treg cells with features of Th1 cells. Indeed, the ability of Treg cells to adopt features of effector T cells is critical for these to cells to control inflammation mediated by those cells in the periphery (Koch et al., 2009).

In addition to regulating Th1 plasticity, analysis of RARα binding in Th17 cells identified RA regulation of Th1 associated genes, such as *Tbx21* and *Il12rb2*, suggesting that RA signaling might be required for the plasticity of Th17 cells in response to a Th1-polarising stimulus. Such plasticity has been observed in vitro and in vivo and is associated with autoimmune disease pathogenesis (Basu et al., 2013).

Distinct mechanisms of gene regulation by RARα

The ability of RARα to repress alternative cell fates highlights the importance of gene repression by RA/RARα. A number of genes that were bound by RARα were transcriptionally repressed. Whilst our data provides a clear mechanism by which RA/RARα positively regulates gene expression, it remains unclear how RARα represses genes that instruct alternative cell fates to enforce lineage stability. RA signaling through RARα can also induce binding of co-repressors (e.g. RIP-140) to the AF-2 domain. It is unclear what factors determine binding of a coactivator complex vs. a corepressor complex to RARα. Differential effects of ligand-dependent receptors on transcription has previously been demonstrated (Diamond et al., 1990). Further analysis of the binding sites that mediate ligand-dependent inhibition of genes and those that mediate positive transcription by RARα may reveal distinct binding partners that determine the differential effects of RARα. An unexpected observation was that RARα binding at promoters has no effect on gene transcription in the absence of RARα binding at its presumed enhancer. It is unclear, in this case, what role RARα plays at the promoter. We speculate that RARα may act as a signal sensing

scaffold that regulates chromatin interactions between promoter and enhancer. Thus, promoter bound RARα only becomes relevant once its cognate enhancer is activated.

Interplay of RARα and Th1 lineage defining transcription factors

T-bet, STAT4 and STAT1 have also been shown to drive active enhancers in Th1 cells (Vahedi et al., 2012). We identified a high degree of overlap between the sites occupied by RARα and lineage-determining transcription factors (LDTFs) in Th1 cells. Previous studies have focused on identifying individual TFs that may account for enhancer activation. However, it is clear that enhancers are platforms for multiple TFs and these factors contribute to enhancer activation, often in a cooperative manner. Enhancers may appear to be dependent on one transcription factor, as a result of the hierarchy in TF binding, whereby Factor A recruits B which in turn recruits C. In this model, loss of Factor A would prevent enhancer activation but A, B and C are all required for a fully functioning enhancer. A is necessary but not sufficient. Whilst STAT proteins may be critical for enhancer activation, it is possible that they operate through RARα.

Importantly, our analysis identified independent effects of RARα on p300 recruitment, suggesting that RARα is an additional layer of the machinery required for the activation of Th1 enhancers. The combinatorial effects of RARα along with T-bet, STAT1 and STAT4 on p300 binding at enhancers suggest a complex layer of regulation allowing enhancers to integrate extracellular cues received from both cytokines and RA. The different combinatorial patterns of transcription factor binding at active enhancers suggest that some enhancers

may require multiple signals to become fully active. Strikingly, amongst key Th1 cell regulators, RARα appeared to have the greatest effect on p300 recruitment.

An intriguing finding that warrants further investigation is the synergistic relationship of RARα and T-bet on p300 recruitment. Possibly, allosteric interactions between T-bet and RARα may facilitate binding of p300 to the respective proteins. Thus, in addition to regulating the expression of Th1 LDTFs, as demonstrated in **Chapter 2**, it appears that RARα acts post-translationally in co-operation with these transcription factors to generate active enhancers.

RARα is selectively recruited to lineage specific enhancers

RARα regulation of naïve $CD4^+$ T cell fate is not restricted to Th1 cells. Comparison of enhancers across naïve, Th1 and Th17 cells allows a temporal analysis of cell-specific enhancers. Our data indicate that RARα is selectively recruited to lineage-specific enhancers, suggesting that LDTFs may guide RARα targets. This selective recruitment provides a mechanistic basis for the pleotropic effects of RA on T-cell fate. RARα occupancy overlapped with combinations of Th1 defining transcription factors rather than one particular transcription factor. Thus, it remains unclear how RARα is recruited to these sites, and whether indeed there is a single, responsible factor. Motif analysis of the Th1 lineage-specific RARα bound enhancers did not reveal a specific co-factor, but indicated the presence of a number of candidate pioneer factors, e.g. AP-1 and RUNX. Further studies are required to confirm co-occupancy. It is unclear whether these factors recruit RARα in order to activate enhancers, or

RARα modulates chromatin in order to allow these TFs to bind. Nearly all RARα bound sites were occupied by p300 with very few RARα bound enhancers marked solely by H3K4me1. In addition, RARα was not required for H3K4me1 deposition at enhancers. This suggests that RARα is recruited to permissive enhancers downstream of pioneer factors. Dynamic ChIP-seq studies for these transcription factors during Th1 differentiation will allow greater temporal resolution of the order of binding events at Th1 enhancers, and shed light on the step-wise processes that generate active enhancers.

It has been suggested that RARα forms part of a multi-protein complex that regulates enhancer activity through recruitment of co-regulators (Bush et al., 2003; Liu et al., 2014). Our data support such a model, providing evidence that p300 recruitment is critically dependent on the presence and activation of RARα at enhancers.

RA/RARα regulation of Th17 enhancers

The data in chapter 3 provide a mechanistic basis for the paradoxical effects of RA on Th17 differentiation, demonstrating cell-context dependent actions of RARα at Th17 related genes. During both Th1 and iTreg differentiation, RA/RARα signaling has been shown to repress Th17 lineage development (Brown et al., 2015; Mucida et al., 2007). However, studies with vitamin A deficient mice have suggested that RA is also required for Th17 generation at mucosal sites both in steady state and in response to inflammation (Hall et al., 2011; Uematsu et al., 2008). These studies were performed in vitamin A deficient mice and it was not clear whether there was a cell intrinsic role for

RA/RARα in the generation of Th17 responses. Comparison of RARα binding in differentiating Th1 vs. Th17 cells showed overlapping but distinct targets and initial results suggest that RARα signaling is required for stable RORγt and IL-17A expression in Th17 cells. The cell context dependent effects of RARα may be mediated in part through expression of distinct co-factors amongst $CD4^+$ T cells. Further analysis of the cell-specific RARα bound regions will shed light on potential lineage-specific co-factors.

Transcription factor binding does not necessarily equate with transcriptional changes and further studies are required to confirm the in vivo relevance of RARα regulation of Th17 loci and identify the circumstances in which RA regulates Th17 responses. Th17 cells represent a heterogeneous lineage and these cells display considerable early and late phase plasticity. RARα binding at the *Tbx21* and *Ifng* loci in both Th1 and Th17 cells suggests that RA may regulate the conversion of Th17 cells to IFN-γ^+IL-17^+ cells. Transcriptional profiling of dn*Rara* Th17 cells along with studies of their stability/plasticity are required to dissect out the role of RA in the regulation of Th17 differentiation.

Translational significance

There are at least two ways in which an understanding of the spatiotemporal, cell-context specific actions of RARα is critical for translation of these findings into clinical practice. Firstly, they may help explain a paradoxical observation that has arisen from the use of Vitamin A supplementation in areas where vitamin A deficiency is endemic. Although hailed as a success, meta-analysis of

vitamin A supplementation in children has identified sub-groups in whom routine supplementation is associated with increased morbidity and mortality. We can now speculate that, since RA/RARα may enhance effector immune responses, the administration of RA during such a response (e.g. an acute infection) may be inadvertently harmful. Secondly, the RA/RARα signaling axis is a therapeutic target for a number of selective RARα agonists in development. These have a potential role both in autoimmune disease, in which dysregulated Th1/Th17 responses are observed, and for enhancing Th1 effector responses, e.g. as an adjuvant in tumour immunotherapy. However, it is apparent from studies in this chapter that the choice and use of agonist will depend on whether the desired response is repression or activation, and requires p300 recruitment, ligand blockade, or as yet unidentified protein interactions.

In summary, our results identify global effects of RA/RARα on epigenetic landscape in T helper cells and demonstrate cell context-dependent effects of RARα on lineage programming.

CHAPTER 4: CONCLUSION

RARα maintains Th1 lineage commitment in vitro and in vivo

My investigations have redefined the role of RARα in T helper cell specification. Prior to these studies, RARα had been shown to enhance iTreg generation in vitro, and a role for RARα in Th1 responses in vivo had been suggested. But the mechanistic basis for the cell-specific actions of RA in the regulation of T-cell fate has remained elusive. Initial reports concluded that RARα played a role in T cell activation. However, these studies were performed in RARα$^{-/-}$ mice which may not be an appropriate genetic model for loss of RA signaling. Although RARα is known to be a signal-dependent transcription factor, no previous study had explored the genome wide regulation of CD4^{+} T-cell programs by RARα. The studies presented in chapter 2 show that RA signaling through RARα is critical for maintenance of Th1 commitment. The ChIP-sequencing studies in both chapters 2 and 3 show that RARα positively regulates the transcription of key Th1 factors to reinforce lineage stability, whilst antagonizing alternative T helper cell programs.

The requirement for RA signaling for late phase Th1 commitment, but not lineage specification, may be explained by the finding that RARα regulates expression of Th1 lineage defining transcription factors by activation of cis-regulatory enhancer regions. Enhancer regions are activated during the course of T-helper cell differentiation (Hawkins et al., 2013), and it has been suggested that the role of enhancers is to sustain rather than initiate gene transcription. Recruitment of RARα to enhancers may be dependent on transcription factors

whose expression is upregulated during the course of Th1 differentiation. The temporal effects of RA may explain findings from previous in vitro studies in which bi-phasic effects of RA were noted during Th1 polarization. Animal models which allow temporal control of RA signaling will be invaluable for dissecting out the role of RA in early *vs*. late phase differentiation.

The discovery that Th1 cells require RA signaling to maintain their phenotype upon reactivation may have implications for therapeutic strategies in autoimmune disease where a dysregulated Th1-Th17 axis plays a role in disease pathogenesis. Our data support a model whereby therapeutic administration of RA would drive differentiation of Th1-Th17 cells to terminally differentiated Th1 cells with reduced survival in contrast to their Th17 counterparts (Muranski et al., 2011). In support of this therapeutic strategy, a recent study showed that RA treatment of $CD4^+$ T-cells from patients with ankylosing spondylitis resulted in a reduced frequency of IL-17+ cells and a concomitant increase in IFN-γ^+ cells (Bidad et al., 2013).

Novel players in the regulation of Th1 stability

Early studies of Th1 regulators identified transcription factors that were both necessary and sufficient for the Th1 phenotype. Advances in transcriptional profiling have expanded the network of transcription factors expressed by different T helper cell subsets (Ciofani et al., 2012). It is increasingly evident that commitment and maintenance of a particular T-helper cell phenotype is dependent on the interplay between numerous transcription factors. Analysis of RARα-bound loci in Th1 cells in parallel with RA dependent gene expression

profiling uncovered novel candidates. Our results in Chapter 2 demonstrated that IRF8 forms part of the transcriptional network in Th1 cells and is critically dependent on RA signaling. Although previously shown to play a role in suppressing IL-17 in Th17 cells, a role for IRF8 in Th1 cells had not been established. Shortly after publication of this work, another group published a study corroborating our findings (Lee et al., 2015), demonstrating selective expression of IRF8 in Th1 and Tr1 cells and a role for IRF8 in the expression of Th1 associated genes in Treg cells.

Functional analysis of Th1 specific RARα bound regions highlighted a number of key transcriptional pathways that may be dependent on RA signaling, including metabolic pathways. RA-mediated regulation of these pathways warrants further investigation.

RA/RARα confers stability to T helper cell commitment

RA synthesis occurs at sites of T-cell priming in response to a diverse array of stimuli. In the steady state, constitutive expression of RALDH isoforms by APCs and stromal cells is observed at barrier sites including the skin, lung and gut. It is perhaps not surprising, given the importance of RA in regulating T cell fate, that steady state production of RA occurs at mucosal sites where T cells are continuously differentiating in response to commensals and other innocuous antigens. In keeping with this, microbial products have been shown to induce the expression of *raldh* isoforms (Singh et al., 2014). Outside of these sites, RA synthesis can be induced by inflammation as demonstrated in a number of in vivo models (Allie et al., 2013; Hall et al., 2011; Pino-Lagos et al., 2011). Our

studies have now shown the fundamental importance of an RA signal during T cell priming. The data demonstrated that RA signals during T helper cell differentiation are required for enhancer activation to reinforce initial lineage specification. In this way RARα, a nuclear receptor, bridges the extracellular environment with transcriptional and epigenetic machinery. Our studies have focused on regulation of Th1 enhancers. However, the data presented in Chapter 3 suggest a pervasive role for RA/RARα in the generation of active enhancers in alternative T helper cell subsets. Multiple signals are required to activate a T helper cell differentiation program. It now appears that RA signaling is also required to stabilize initial lineage specification through epigenetic modifications. We propose that, in addition to TCR engagement, co-stimulatory signal and cytokine receptor signal, an RA/RARα signal is a further checkpoint that acts to link the extracellular environment to transcriptional changes by licensing the recruitment of p300 and other factors which regulate H3K27 acetylation. A continuous requirement for RA signaling provides flexibility to T helper cells, allowing RA gradients to regulate T cell plasticity in response to changes in the extracellular milieu.

Dissecting the multi-step process required for generation of cell-specific enhancers

Given the fundamental importance of enhancer regions in determining cell fate, the mechanisms responsible for enhancer activation and function have been the focus of intense investigation. Despite advances in our ability to globally map enhancer regions, mechanistic understanding of the processes required to confer epigenetic changes remains elusive. Studies in models of cellular

differentiation indicate that there is a precise and hierarchical temporal order of transcription factor binding and co-factor recruitment. This process is initiated by the binding of pioneer transcription factors. Chromatin accessibility then allows binding of lineage-specific factors to these regions. Our findings integrate RA signals into this multi-step process. RA is not required for initiation of enhancers suggesting that alternative pioneer factors are required before RARα can activate enhancers through the recruitment of p300. Despite the expression of RARα by naïve T cells, effects of RA on T-cell polarity are only observed following T-cell activation and differentiation. This is consistent with a model in which TCR induced pioneer factors act upstream of RA signaling.

An emerging view – RARα, a central component of the enhanceosome

This is the first study to compare genome wide RARα binding between different T helper cell lineages. In this study, we resolve paradoxical findings regarding the role of RA in Th17 differentiation and provide a molecular mechanism that explains previous studies. Our data provide a mechanistic basis for the pleiotropic roles for RA in the generation of Treg, Th1, Th17, and Th2 cells, suggesting that RA signaling is not lineage-specifying but rather acts to reinforce cell fate decisions through epigenetic mechanisms. There are two ways in which the ability of RARα to regulate gene expression in a cell type-specific manner might be determined. One is that lineage specific factors recruit RARα, in *trans*, to pre-selected enhancers, where it acts as a co-factor, recruiting p300. The alternative is that RARα binding to RARE cis-binding sites is facilitated by lineage specific pioneer factors which determine the accessibility of RAREs within enhancer elements. Analysis of RARα binding in cells

expressing mutant RARα, lacking the DNA binding domain, could distinguish between these two possibilities. In either model, rather than specifying T-cell fate, RA enhances or stabilizes the actions of cytokines and transcription factors that guide T-helper cell programming.

Based on the data above, we propose a model whereby RARα acts as the bridge between external environmental cues and the chromatin modifiers but its site of action and thus its specificity is determined by lineage-specific factors such as lncRNAs or lineage-defining transcription factors. We hypothesise that lineage-specific factors may act as cofactors, either directing recruitment of RARα to permissive cell-type specific enhancers and/or licensing its action at sites of binding. An analogous role has been identified for the nuclear hormone androgen receptor where a number of lncRNAs were shown to license androgen receptor activity at enhancer regions (Hsieh et al., 2014). Temporal analysis of lncRNA expression during T helper cell specification has revealed cell-type specific patterns of expression, and highlighted a number of lncRNAs which are potential candidates for recruitment of RARα to lineage-specific enhancers in T cells. The feasibility of identifying the protein target of a candidate lncRNA has recently been reported, in studies of *Xist*, the lncRNA responsible for X-inactivation (McHugh et al., 2015). Investigation of potential interactions between RARα and lineage specific lncRNAs is an area of future work.

Given that RARα has been implicated in the differentiation of a number of cell types, further studies are required to address the broader relevance of

our findings in cell lineages outside of the immune system. In support of a global role for RARα in the regulation of enhancers, a recent study identified RARα as part of a complex of transcription factors, that were critical for the regulation of oestrogen-receptor dependent enhancers. In this study, RARα was recruited in *trans* to enhancer regions and the specificity of its actions at the genome level were determined by the oestrogen receptor. Given that RA/RARα is a highly conserved signaling pathway, which plays a critical role in regulating cell fate specification during embryogenesis and cell differentiation, it is perhaps not surprising that RA has been implicated in the fate of multiple T helper cell subsets.

Retinoic acid – an immune morphogen?

An additional aspect of RA regulation of T cell fate that remains to be addressed is the dose dependent effect of RA on T cell fate. Similar to the morphogenic properties of RA during embryogenesis, the RA concentration sensed by naïve T-cells undergoing differentiation determines the dominant action of RA on T-cell fate. Over the past few years, RA has achieved recognition as a morphogen, as it has become clear that synthesis and metabolism of RA is tightly controlled resulting in concentration dependent effects on target tissues (Casci, 2008). The preponderance of data from *in vitro* experiments in which dose titration comparisons were performed on Th1, Th2 and Th17 polarisation suggest similar dose dependent effects of RA on haematopoietic cell fate (Iwata et al., 2003; Takahashi et al., 2012; Uematsu et al., 2008). The paradoxical effects of RA on opposing T-cell fates may therefore be explained by concentration dependent effects of RA, allowing T-cells to act

as an environmental sensor through the strength of its RA signal. Further studies of RA gradients within lymphoid tissue as well as dose dependent effects of RA on RARα recruitment and p300 occupancy are required to test this hypothesis. However, the overriding message is that administration of RA either in vitro or to vitamin A replete hosts in vivo may not provide insight into the physiological actions of RA on T-cell responses.

By blocking endogenous ligand signaling through RARα, rather than studying effects of added RA, my experiments sought to study physiological RA signaling, and did not address questions of concentration dependence. In vitro, the likely source of RARα agonists are RA and retinol present in serum. RXR agonists also activate the AF-2 domain of RARα. The endogenous ligands for RXR remain elusive. A number of molecules can bind to the RXR lipid binding pocket, including long chain unsaturated fatty acids such as omega-3 fatty acid docosahexaenoic acid (DHA) and other naturally occurring polyunsaturated fatty acids (Lengqvist et al., 2004). Recent studies have shown that extensive metabolic reprogramming accompanies T helper cell differentiation, including changes in cellular lipid metabolism (Pearce et al., 2013). It is possible that a shift in metabolism generates intermediaries which serve as endogenous intrinsic RXR agonists signaling through RAR/RXR heterodimers. Intriguingly inhibition of fatty acid synthesis was shown to significantly impair Th17 responses as well as Th1 and Th2 differentiation (Lochner et al., 2015). It is tempting to speculate that synthesis of RXR ligands by effector T cells during differentiation provides an autoregulatory pathway that sustains stable expression of gene expression through rounds of cell proliferation.

Concluding remarks

In summary, this work provides the first mechanistic framework for the regulation of T helper cell differentiation by RARα and sheds light on the fundamental principles by which enhancers are activated. By coupling RARα, a ligand-dependent transcription factor, which senses local changes in the microenvironment, to regulatory chromatin regions, RARα signaling contributes to dynamic changes in enhancers, regulating the stability and plasticity of T helper cell lineages. T cells represent an excellent model system for gaining insights into how extracellular cues can direct changes in chromatin structure and how this translates into stable phenotypic changes.

CHAPTER 5: Materials & Methods

General Methods

Mouse strains

C57Bl/6 (WT) were purchased from Charles River, UK. *Cd4*^{cre}, and *Ifng*^{eYFP} (GREAT) mice were purchased from the Jackson Laboratory. *Rosa26*$^{dnRara/dnRara}$ mice were originally obtained from Dr. Sockanathana (Rajaii et al., 2008). F1 progeny of *Cd4*^{cre} and *Rosa26*$^{dnRara/dnRara}$.breeders were first backcrossed onto the C57Bl/6 background from Jackson. *Cd4*^{cre}*Rosa26*$^{dnRara/dnRara}$ (dn*Rara*) mice were subsequently generated by crossing *Cd4*^{cre}*Rosa26*^{dnRara} and *Rosa26*^{dnRara} breeders. dn*Rara* mice have been described previously (Pino Lagos). Mice were bred and maintained at Charles River Laboratory, UK, or Kings College London, UK, in pathogen-free conditions. All animal experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986. C57Bl/6 OTII(dn*Rara*), OTII and *Rag1*$^{-/-}$ mice were bred and maintained at the Rockefeller University specific pathogen free animal facility. All mice were used between 6-12 weeks of age. In each experiment, mice were littermate or gender and age matched.

Reagents

$LLO_{190-201}$ was synthesised by PiProteomics and was >95% pure, as determined by HPLC. LLO:I-A^b monomers were provided by NIH Core Tetramer Facility. PE labeled LLO:I-A^b dextramers were synthesised by Immudex.

Recombinant Lm-2W strain was provided by Marc Jenkin's Laboratory. LE540 was purchased from Alpha Laboratories and resuspended in DMSO (Sigma)

Naïve $CD4^+$ T-cell isolation

Spleens were harvested and mashed through a 70μm filter to obtain single cell suspensions. Cells were washed and enriched for $CD4^+$ T cells using a $CD4^+$ T-cell negative selection kit (Miltenyi Biotec). Enriched cells were first blocked with 1:100 anti-CD16/32 (2.4G2, eBioscience) and then labeled with a monoclonal antibody (mAb) cocktail containing anti-CD4 (RM4-5), anti-CD25 (PC61), anti-CD62L (MEL14) and anti-CD44 (IM7). Naïve $CD4^+CD25^{neg}CD44^{lo}CD62L^{hi}$ T-cells were purified by cell sorting using a FACSAria (BD). In experiments where eYFP reporter strains were used, $eYFP^+$ cells were excluded.

Antigen Presenting Cell (APC) isolation

T-cell depleted splenocytes were used as APCs. Spleens were harvested, cut into sections and perfused with collagenase (50mg/ml) and DNase I (20mg/ml) in warmed RPMI-1640. Tissues were placed in 5ml of perfusion media per spleen, and digested for 45 minutes at 37°C. Spleen fragments were then mashed through a 70μm filter to obtain single cell suspensions, washed with complete RPMI-1640 (cRPMI) medium containing 10% fetal bovine serum (FBS), 50 μM β-mercaptoethanol, 25mM HEPES, non-essential amino acids, glutamine and 100μg/ml penicillin/streptomycin. Cells were depleted of T cells using a $CD3^+$ microbead selection kit (Miltenyi Biotec). Purity was checked and always exceeded 98%. Depleted cells were resuspended in cRPMI at 2 $x10^6$/ml and irradiated at 3000 rad.

Naïve CD4$^+$ T-cell differentiation

Naïve CD4$^+$ T-cells were cultured for 3 days with irradiated T cell-depleted splenocytes at a ratio of 1:5 in the presence of 5 μg/ml of anti-CD3 (145-2C11) under Th0 cell conditions (IL-2 100 IU/ml, anti-IL-4 (11B11) and 10 μg/ml anti-IFN-γ (XMG1.2); Th1 cell conditions (100 IU/ml of IL-2, 10 ng/ml of IL-12, and anti-IL-4 10 μg/ml); Th2 cell conditions (100 IU/ml of IL-2, 10 ng/ml of IL-4, anti-IL-12 (C17.8), and 10 μg/ml of anti-IFN-γ (XMG1.2); or Th17 cell conditions, 5 ng/ml TGF-β, 20 ng/ml IL-6, 10 ng/ml IL-1β, 10 μg/ml of anti-IL-4, and anti-IFN-γ). Cells were expanded for an additional 3-4 days. IL-2 was supplemented in cultures every 2 days with the exception of Th17 cultures. Where indicated, 10 ng/ml IFN-γ or 10 μg/ml anti-IFN-γ was added. In secondary repolarisation assays, where specified, LE540 (1 μM) or DMSO (vehicle control) was added to the media. IL-2 was from Miltenyi and other cytokines were from R&D Systems. Anti-CD3 was from BioXcell and other antibodies were from BD Biosciences. All cell cultures were performed in complete RPMI.

Flow Cytometry

For analysis of cytokine production, cells were restimulated with phorbol 12-myristate 13-acetate (PMA) 100 ng/ml and ionomycin 500 ng/ml in the presence of monensin (1μl/ml Biolegend) for 4-5 h at 37^{o}C in a tissue culture incubator (37^{o}C/5%CO_2). Cell surface staining was carried out in PBS with 2% FBS. For live cell analysis or cell sorting, dead cells were excluded by staining with SYTOX blue (Invitrogen). For intracellular staining, cells were first stained with LIVE/DEAD^{TM} Fixable Violet or near IR Dead Cell Stain (Invitrogen) in PBS and blocked with 1:100 anti-mouse CD16/32, followed by staining for cell-surface

markers and then resuspended in fixation/permeabilisation solution (Cytofix/Cytoperm kit or Transcription Factor Buffer kit; BD Biosciences). Intracellular staining was carried out in accordance with the manufacturer's instructions. The fluorescent-dye-conjugated antibodies used were obtained from BD-Biosciences: anti-CD4 (RM4-5), anti-CD3 (145-2C11); anti-CD62L (MEL14), anti-CD44 (IM7), anti-CD8α (53-6.7), anti-IL-17A (TC11-18H10), anti-IFN-γ (XMG1.2), anti-IL-4 (11B11), anti-RORγt (Q31-378); eBioscience: anti-Foxp3 (FJK-16a), anti-T-bet (eBio4B10) or Biolegend: anti-CD25 (PC61). The following isotype controls were included in staining panels where indicated: rat IgG1, rat IgG2a, mouse IgG2a, and mouse IgG1. AccuCheck counting beads (Invitrogen) were added to samples for calculation of absolute cell numbers. Data were collected with a LSR Fortessa (BD) and results were analyzed with FlowJo software (Tree Star).

PhosphoSTAT staining

Intracellular phosphorylated STAT proteins were stained with Phosflow Lyse/Fix Buffer, and Phosflow Perm Buffer III (BD Biosciences) according to the manufacturer's protocol. The following antibodies were used for detection of phosphorylated STAT proteins: anti-pSTAT1 (pY701), anti-pSTAT4 (pY693) and anti-pSTAT3 (pY705). All antibodies were obtained from BD-Biosciences.

Chromatin immunoprecipitation (ChIP)

Prior to harvesting, dead cells were depleted from culture with the Dead Cell Removal kit (Miltenyi Biotec) as per manufacturer's protocol. 20-60 million Th1 polarised cells from WT and dn*Rara* mice were fixed, washed and snap-frozen

according to the Cell Fixation protocol from Active Motif. Cells were resuspended in complete RPMI and fixed with the addition of 1/10 volume of freshly prepared Formaldehyde solution containing 11% Formaldehyde (Sigma), 0.1M NaCl (Sigma), 1mM EDTA (Sigma0 and 1M HEPES (Affymetrix). Cells were agitated for 15 minutes at room termperature. 1/20 volume of 2.5M Glycine solution was added to stop the fixation and cells were incubated for 5 minutes. All subsequent steps were performed on ice or at 4^{o}C. Cells were washed once with PBS 0.5% Igepal and a second time with PBS-Igepal containing 1mM Phenylmethanesulfonyl fluoride. Cells were pelleted, snap frozen in liquid nitrogen and stored at -80^{o}C.

Chromatin was isolated by the addition of lysis buffer, followed by disruption with a Dounce homogenizer. Lysates were sonicated and the DNA sheared to an average length of 300-500 bp. Genomic DNA (Input) was prepared by treating aliquots of chromatin with RNase, proteinase K and heat for de-crosslinking, followed by ethanol precipitation. Pellets were resuspended and the resulting DNA was quantified on a NanoDrop spectrophotometer. Extrapolation to the original chromatin volume allowed quantitation of the total chromatin yield. An aliquot of chromatin was precleared with protein A agarose beads (Invitrogen). Following immunoprecipitation with specified antibodies, complexes were washed, eluted from the beads with SDS buffer, and subjected to RNase and proteinase K treatment. Crosslinks were reversed by incubation overnight at 65 C, and ChIP DNA was purified by phenol-chloroform extraction and ethanol precipitation and used for the preparation of Illumina sequencing libraries and for ChIP qPCR analysis.

ChIP-qPCR

Quantitative PCR (qPCR) reactions were carried out in triplicate on specific genomic regions using SYBR Green Supermix (Bio-Rad). See Table S5 for Primer details. The resulting signals were normalized for primer efficiency by carrying out qPCR for each primer pair using Input DNA. By using standards of known quantities of DNA it was possible to calculate the number of genome copies pulled down for each of the sites tested, and thus to calculate the copies pulled down per starting cell number, presented as 'Enrichment'. For RARα ChIP qPCR a gene desert on chromosome 6 (Untr6) was used for a negative control site (Active Motif Catalog No: 71011).

ChIP Sequencing (Illumina)

Illumina sequencing libraries were prepared from the ChIP and Input DNAs using standard procedures and libraries were sequenced on HiSeq 2500 (Chapter 2) or Illumina NextSeq 500 (Chapter 3).

CHAPTER 2 Methods:

Cell Proliferation analysis

Cells were labeled at a concentration of $1x10^6$cells/ml with 5μM CellTrace™ Violet (Invitrogen) diluted in PBS for 20 min at $37^oC/5\%CO_2$. Labeling was quenched with 5X volume of complete RPMI. Cells were incubated for 10minutes prior to washing and resuspending in complete RPMI. Cells were stimulated under Th1 conditions as detailed above.

TAT-Cre transduction

Sort purified naïve $CD4^+$ T cells were differentiated under Th1 conditions. After 5 days, cells were washed twice in serum free medium prior to treatment with 50 μg/ml TAT-Cre (Millipore) or medium alone (mock treatment). Cells were incubated at 37°C for 45 minutes. The reaction was quenched with equal volumes of medium containing 20% FBS followed by further washing. Cells were expanded for 2 days in cRPMI with IL-2 (50 IU/ml), followed by retreatment with TAT-Cre or media as before. Cells were then restimulated under Th1 cell conditions for 3 days and expanded for a further 2 days prior to analysis. IL-2 (50IU/ml) was supplemented in cultures every 2 days.

Western Blotting

Differentiated Th1 cells were lysed in RIPA buffer supplemented with protease inhibitors. Lysates were electrophoresed on 10% gels (Biorad), transferred to nitrocellulose and blotted with anti-STAT4 or anti-actin followed by anti-rabbit-horseradish peroxidase conjugated antibody. All antibodies were from Cell

Signaling Technology.

L. monocytogenes (actA LM-2W) infection

L. monocytogenes was grown overnight in LB broth supplemented with chloramphenicol (20µg/mL), in a shaker at 37°C, 250rpm. After 12-16hrs, the bacteria were sub-cultured after a 1:100 split and grown to an OD_{600} of ~0.1. Bacterial cells were harvested at 5,000 g for 10 min and resuspended to $5x10^6$ cfu/ml in room temperature PBS. Mice were infected intra-venously via tail vein injection with 1×10^6 cfu.

L. monocytogenes analysis

For FACS analysis, single cell suspensions of spleens from *L.monocytogenes* infected mice were prepared as described above. Red blood cells were lysed in ACK for 2 minutes at room temperature. Single cell suspensions were enriched for $CD4^+$ T-cells with a $CD4^+$ T-cell negative selection microbead kit (Miltenyi Biotec) and stained with PE labeled, LLO:I-A^b dextramer (Immudex) in complete RPMI for 1 h at 37°C/5%CO_2. Cells were then transferred to ice and stained with cell surface antibodies. The antibodies used were obtained from Ebioscience: anti-CD3 (145-2C11), anti-B220 (RA3-682), anti-CD11c (N418), anti-CD11b (M170), anti-F4/80 (BM8), or BD Bioscience: anti-CD4 (RM4-5) anti-CD44 (IM7) anti-CD8α (53-6.7) or Biolegend: anti-IL6-Rα (D7715A7).

In vitro recall to *L. monocytogenes*

Single cell suspensions of spleens from *L.monocytogenes* infected mice were prepared as described above. For analysis of cytokine production, supernatants

were collected from splenocytes restimulated with LLO peptide (PiProteomics) at 10 µg/ml for 24 h. For analysis of intracellular cytokines, splenocytes were stimulated with LLO peptide for 6 h in the presence of monensin. Intracellular cytokine staining was performed as described above.

Luminex Immunoassays

Cytokine levels in supernatants were measured using a multiplex bead-based assay (Bio-Rad Laboratories) in a Luminex FlexMap3D System (Luminex Corporation) as per the manufacturer's instructions.

Real-Time Quantitative PCR.

Total RNA was extracted from cells with RNeasy Mini kit (Qiagen) and cDNA was synthesised with Qscript RT kit (Quanta). Quantitative gene expression analysis was peformed using Taqman primer probe sets (Applied Biosystems), listed in Table S4 of Chapter 2. Expression of target genes was normalized to b-actin.

ChipSeq Analysis

For each sample the 50bp SE reads in FastQ format from the sequencer were aligned to the mouse reference genome (mm10) using Novoalign v2.07.11 (http://www.novocraft.com). The resulting alignment file was converted to BAM format using samtools (http://samtools.sourceforge.net/) and the PCR duplicates were removed using picard tools (http://picard.sourceforge.net). Only uniquely mapped reads from each sample were selected for further analysis. Significantly enriched regions from each sample were identified with MACS

v2.0.10 (Feng et al., 2011; Zhang et al., 2008) (with q=0.10) using the input sample for background correction. In some instances peaks were identified by visual inspection and confirmed by ChIP qPCR. In case of H3K4me1 and H3K27me3 samples, "--broad" setting was used to merge nearby enriched regions. For visualization purposes, the input signal was subtracted from each ChIP sample and was converted into bigWig format using "bedGraphToBigWig" utility from UCSC tools (http://genome.ucsc.edu/util.html). The identified significantly enriched regions were annotated to find the associated genes using "FindNeighbouringGenes" utility from USeq package (http://useq.sourceforge.net/). Associated genes represent the closest transcriptional start site from the centre of the peak.

Microarray data

Total RNA was extracted from cells lysed in Trizol LS reagent (Life Technologies). RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies) and quantified with the Nanodrop ND-1000 UV-spectrophotometer (NanoDrop Technologies).

Transcriptome in IFN-γ^+ (eYFP$^+$) CD4$^+$ T-cells

Naïve CD4$^+$ T-cells from dn*Rara*-IFN-γ^{eYFP} or littermate control IFN-γ^{eYFP} reporter mice were cultured under Th1 conditions. On day 7 of culture, following restimulation with PMA and ionomycin, eYFP$^+$ cells were sorted and total RNA was extracted for transcriptional profiling using Affymetrix Mouse Gene 2.0 ST arrays. Pre-processing and statistical analysis of gene expression data were done using Partek Genomics Suite 6.6. CEL files were imported and expression intensities were summarised, normalised and transformed using Robust

Multiarray Average algorithm. Two additional samples from eYFP$^+$ dn*Rara* or wild-type cells sorted without prior restimulation were included in the normalisation. These samples were not included in the analysis of differentially expressed genes. P values <0.05 and fold change in expression ≥ 1.5 or ≤ -1.5 were considered significant.

Transcriptome in Th1 differentiated cells

Sorted naïve CD4$^+$ T-cells from dn*Rara* or WT mice were polarised under Th1 conditions. On day 6 of culture cells were harvested and total RNA was extracted for microarray study or ChIP. RNA isolation, microarray and data processing performed by Miltenyi Biotec. Transcriptome analysis was performed using Agilent Whole Mouse Genome Oligo Microarrays 8X60K in accordance with manufacturer's protocol. Data analysis was performed using R/bioconductor and software packages therein (http://www.R-project.org ; http://www.bioconductor.org) or MS-Office Excel (Microsoft Inc.). Background corrected intensity values were normalized between arrays using quantile normalization. Quality controls include comparison of intensity profiles and a global correlation analysis. Differentially expressed genes were identified by statistical group comparisons on normalized (background corrected and quantile normalized) log2 transformed fluorescence intensities using Student´s t-test (two-tailed, equal variance). Reporters showing a p-value ≤ 0.05 and a median fold-change in expression ≥ 1.5 or ≤ -1.5 were considered as reliable candidates for altered gene expression. In addition, at least two of the replicate samples in the group with higher expression were required to have detection p-values ≤ 0.01.

CHAPTER 3 Methods

Th17 cells for ChIP

Th17 cells were harvested on day 5 of culture and prepared as outlined above in 'General Methods – ChIP'. For analysis of Th17 cells on day 3 of culture, cells were Fc blocked and labelled with anti-CD4 (RM4-5) and SYTOX to exclude dead cells. Live $CD4^+$ T cells were purified by cell sorting using a FACSAria (BD). Cells were then fixed in accordance with Active Motif's protocol, as detailed above.

Microarray data

Transcriptome data from dn*Rara* and WT Th1 cells generated in Chapter 2 was analysed in R/bioconductor using the limma program package. Background correction and quantile normalization was performed. Control probes and low expression probes as well as lincRNAs and probes with unknown annotations were filtered. Differentially expressed genes (fold change >1.5, FDR < 0.05) were identified using limma. To determine enrichment of genes up- or downregulated in dn*Rara* Th1 cells at RARα bound promoters and enhancers, we first generated 'gene-sets' based on genes containing RARα peaks at promoter regions (-4kbp to +500bp) (Pro), enhancers (E) or both. Enhancers were associated to the gene closest to the genomic region. Gene sets were filtered by genes that are measured by the Agilent array. Fisher's exact test was used to calculate the significance of the overlap.

Identification of T helper cell subset signature genes

Publicly available gene expression data for Th17, Th1, Th2 and iTreg cells from (Wei et al., 2011) was downloaded and analysed using the default settings in Partek Genomics Suite 6.6. To avoid spurious fold changes due to low expression values, a small constant (c=0.25) was added to the normalized RPKM values. For each subset, we identified genes that were over-expressed compared to all other cell subsets, with a fold change ≥ 1.5 and p value < 0.05. 200 genes were selected at random (excluding genes in the T helper cell subset specific lists). The TSSs of these Th-cell-subset-specific and random genes were selected using RefSeq annotation and extended ±20kb. Normalized tag intensity of Th1 RARα binding was calculated in these extended regions from all cell types and plotted using R. A two-tailed student t-test was performed to calculate the significance of enrichment.

For analysis of Th17-specific RARα bound regions we used a set of literature-curated Th17 genes (Gagliani et al., 2015).

Functional enrichment analysis of bound or affected genes

Th1-specific RARα binding sites were identified as detailed above. The identified regions were annotated to find the associated genes using "FindNeighbouringGenes" utility from USeq package (http://useq.sourceforge.net/). KEGG enrichment for this gene set was carried out using Web-Based Gene Set Analysis Toolkit (Webgestalt) (Zhang et al., 2005). GO enrichment was performed with PANTHER using annotations based on experimental evidence (Thomas, 2003). Functional enrichment analysis of genes containing cell-type-specific RARα binding sites or differentially

expressed genes in dn*Rara* Th1 cells was performed using annotations and datasets from the MSigDB database (Subramanian et al., 2005).

ChIP-seq analysis

The SE reads in FastQ format from each sample were aligned to the mouse reference genome (mm10) using Novoalign v2.07.11 (http://www.novocraft.com). The resulting alignment file was converted to BAM format using samtools (http://samtools.sourceforge.net/) and the PCR duplicates were removed using picard tools (http://picard.sourceforge.net). Only uniquely mapped reads from each sample were selected for further analysis. Significantly enriched regions from each sample were identified with MACS v2.0.10 (Feng et al., 2011; Zhang et al., 2008) using a q value of 0.05. The control library for peak-calling was the input DNA performed under Th1 conditions without antibody for background correction. T-bet binding from publicly available data in (Gökmen et al., 2013) was similarly analysed with its corresponding input. For H3K27me3 libraries , "--broad" setting was used to merge nearby enriched regions and the q-value was set to 0.10. Intensities of all binding sites are normalized to the number of uniquely mapped reads after PCR duplicate removal. For visualization purposes the input signal was subtracted from each ChIP sample and each file was converted into bigWig format using "bedGraphToBigWig" utility from UCSC tools (http://genome.ucsc.edu/util.html). The y-axis in all gene tracks is the normalised read count.

The identified significantly enriched regions were annotated to find the associated genes using "FindNeighbouringGenes" utility from USeq package (http://useq.sourceforge.net/).

Reproducibility of p300 and RARα peaks

The genome was divided into 5kb non-overlapping windows and the normalized tag intensity from each replicate was calculated for each window. All windows with zero intensity in both replicates were removed and all remaining windows were used to plot a correlation between the two replicates. Function "cor" in R was used to compute the r^2 value.

Genomic distribution of RARα binding

The genomic distribution of RARα binding sites was performed using all the sites with a q value ≤ 0.05 identified by the MACS peak caller. The distribution was calculated with "assignGenomeAnnotation" executable within Homer package using RefSeq annotation for the mouse genome assembly mm10.

Delineation of enhancers

Peak calling on duplicates of p300 WT Th1 cells was performed. Peaks that were not common between the two libraries were discarded. All regions that did not overlap with promoter-associated peaks (-4kbp to +500bp of the TSS) were included in the downstream analysis. We then selected regions which overlap with H3K4me1 peaks. To generate the final catalogue of enhancers we further filtered regions based on H3K4me3 intensity to retain regions with no H3K4me3 or low levels of intensity ($H3K4me3^{low}$). To define $H3K4me3^{low}$ regions we first

selected all H3K4me3 regions that overlap with p300 and H3K4me1 peaks outside of promoters and plotted a histogram of the H3K4me3 fold changes at these sites. We observed a bi-modal distribution of H3K4me3 levels. From the distribution we considered H3K4me3 regions with a fold change ≥30 as regions of high H3K4me3 intensity. Removing these regions resulted in 10,511 regions that were considered to be Th1 enhancers. These regions were used for the rest of the analysis unless otherwise stated. Enhancers overlapping with RARα peaks are termed 'RARα bound' enhancers and others as 'RARα negative' enhancers. Overlapping peaks were identified using "intersectBed" from Bedtools.

Identification of Super-enhancers (SEs) and cell-type specific SEs.

Genomic co-ordinates for SE domains in Th1, Th2 and Th17 cell types were downloaded from (Vahedi et al., 2015). The regions were subjected to liftOver to convert to mm10 coordinates. Cell-type-specific super-enhancer regions are defined as regions in one cell condition that have no overlap with any of the other cell conditions, using "intersectBed" from Bedtools with a 1-bp overlap method. The Th1 specific SE regions that overlap with any of the RARα binding sites are defined as RARα-bound Th1 SEs

Binding distribution across a set of coordinates

To check for differences in binding intensity between samples at certain binding sites, the normalized tag intensity of a sample was plotted from the centre of a binding site and extended on either side by 5kb. "sitepro" from CEAS package was used to plot the intensities of uniquely mapped reads after removing PCR

duplicates. For analyais of p300 intensity at Th1 super-enhancers, we used a scale of ±50kb from the centre of each super-enhancer.

Motif Analysis

De novo motif analysis was carried out using the HOMER package. Motifs were searched on the specified peak summit ± 200bp. An equal number of background regions of the same length were randomly selected from the mm10 genome using "randomBed" from Bedtools. In analyses were we compared enrichment of motifs in Th1 specific RARα bound enhancers, Th1 specific RARα binding sites outside of enhancers were used for background regions. We searched for *de novo* motifs (with homer2 executable) for lengths 8 – 14bp using the following parameters (-mis 1 -S 50). HOMER initially identifies oligos of a particular length that are significantly enriched (using binomial distribution) in the target sequences compared to the background. The significant oligos are then converted to position weight matrices. We then compared these significantly enriched PWM's to a dataset of previously known transcription factor PWM's provided within the HOMER collection using "compareMotifs.pl" for each length analyzed. This returns a best identified match from the known transcription factors with a score that relates the identified *de novo* motif to the previously known motif.

Identification of cell-type-specific RARα peaks

Any given RARα binding site is called as specific for a cell type if RARα peaks were observed in one cell type with no overlaps with binding sites in the other cell types. On similar lines we also identified regions that are common between

pairs of cell types or common to all samples. We used “intersectBed” from Bedtools while using “-v” flag to fetch cell-type-specific and common binding sites. The heatmap showing the specific and common regions from naïve, Th1 and Th17 cells was plotted using ngsplot with “-GO none -SC global “ parameters.

Overlap of LDTFs and RARα at Th1 enhancers

We used the RARα and T-bet binding sites identified by our analysis detailed above. For STAT1, STAT4 we downloaded the publicly available binding regions from (Vahedi et al., 2012) and converted to mm10 using liftOver utility from UCSC. For each factor we first removed any binding sites that did not overlap with our Th1 enhancer regions. We then computed the overlap between the remaining binding sites. The venn-diagram was plotted using “diffBind”.

Overlap of RARα binding sites with Foxp3 binding sites

We downloaded the publicly availably binding regions for Foxp3 in Treg cells (Samstein et al., 2012) and converted to mm10. We then computed the overlap with all RARα-bound Th1 enhancers and Th1-specific RARα binding sites.

Regression analysis for p300 intensity

To determine the contribution of different TFs to p300 binding at Th1 enhancers, we performed linear modelling to model the intensity of p300 in the presence or absence of a given TF. To do this we initially calculated the read intensities of p300 in each of the previously described enhancer regions and presence or absence of a factor at each region as binary (0 or 1 for absent and

present respectively). A matrix representing log transformed p300 intensities and the binary representations of factors was prepared and the “lm” function in R was used to calculate the significance of each factors role in explaining p300 intensity, modelling log(p300) as a function of the presence or absence of the TFs. We considered all individual terms, as well as all two-way interaction terms for pairwise combinations of TFs.

Statistical analysis

Statistical significance of the overlap between two groups of genes was calculated using the hypergeometric probability formula.

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